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PROGRAMME

WEDNESDAY 9 JUNE

12.00 – 17.00 Meeting Registration
12.30 Conference Opening

13.00 – 13.40 Opening Lecture
Pier Paolo Pandolfi
Pro-senescence therapy for cancer: from the enhancement of protein translation to the inhibition of protein degradation
Cancer Genetics Program, Beth Israel Deaconess Cancer Center, Department of Medicine and Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA.

13.45 Welcome light lunch

I SESSION - Human and Clinical Proteomics - SIBioC

15.00 – 15.40 Plenary Lecture
Giampaolo Merlini
Proteomics in pathogenesis and diagnosis of systemic amyloidoses
Dipartimento di Biochimica, Università di Pavia, Centro per lo Studio e la Cura delle Amiloidosi Sistemiche, Laboratori di ricerca Biotecnologica, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

15.45 – 16.45 Oral presentations – selected posters
16.45 – 17.00 Coffee Break
17.00 – 18.00 Oral Presentations – selected posters

THURSDAY 10 JUNE

II SESSION - Functional proteomics - SIB

9.00 – 9.40 Plenary Lecture
Stephen Pennington
Quantitative proteomics for biomarkers discovery and validation
University College Dublin - CONWAY Institute of Biomolecular and Biomedical Research – Ireland

9.45 – 10.45 Oral presentations – selected posters
10.45 – 11.15 Coffee Break
11.15 – 12.15 Oral Presentations – selected posters
12.15 – 13.30 Poster session
13.30 – 14.30 Luncheon seminary –**Life technology AB – Sciex**
Proteomics technologies

III SESSION - Plant Proteomics - SIBV

14.45 – 15.30 Plenary Lecture
Alex Jones
Phosphorylation events in plant-pathogen interactions
The Sainsbury Laboratory, Norwich – UK

15.30 – 16.30 Oral presentations – selected posters
16.30 – 16.50 Coffee Break

16.50 – 17.50 Oral Presentations – selected posters
18.00 Members meeting

FRIDAY 11 JUNE

IV SESSION - Technological innovations and Mass Spectrometry - SCI

9.00 – 9.40 Plenary Lecture
Giovanni Sindona
The Specificity of Mass Spectrometry in Biomolecular Research. Present and Future Innovation in Natural and Health Sciences
Dipartimento di Chimica, Università della Calabria ARCAVACATA DI RENDE (CS)-Italy.

9.50 – 10.50 Oral presentations – selected posters
10.50– 11.15 Coffee Break
11.15 – 12.15 Oral Presentations – selected posters
12.15 – 13.30 Poster session
13.30 – 14.30 Lunch

V session - Microbiological and Food Proteomics - SIM

14.45 – 15.30 Plenary Lecture
Maurizio Sanguinetti
Transition from genomics to a post-genomic era in medically relevant Fungi: application of proteomics in clinical diagnosis and biomarker discovery
Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Roma, Italy.

15.30 – 16.30 Oral presentations – selected posters
16.30 – 16.50 Coffee Break
16.50 – 17.50 Oral Presentations – selected posters
20.30 Social Dinner

SATURDAY 12 JUNE

9.00 – 11.00 Oral presentations of selected posters for the "best poster prize"

11.00 – 11.40 Closing Lecture
Joachim Klose
Proteomics and Genomics
Charité Campus Virchow-Klinikum Berlin, Germany

11.45 – 12.00 Closing Remarks

LECTURES

L.1

"PRO-SENESCENCE" THERAPY FOR CANCER: FROM THE ENHANCEMENT OF PROTEIN TRANSLATION TO THE INHIBITION OF PROTEIN DEGRADATION

Pier Paolo Pandolfi
Cancer Genetics Program, Beth Israel Deaconess Cancer Center, Department of Medicine and Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA.

We will present exciting new progress at developing what we regard as a new concept, which we refer to as "pro-senescence therapy", that aims at utilizing cellular senescence, a built-in failsafe mechanism in mammalian cell evoked by oncogenic stress, for cancer prevention and treatment.
We will discuss how this analysis allowed us to identify novel and therapeutically relevant molecular and biological types of cellular senescence that can be evoked and potentiated pharmacologically, in turn allowing the targeting of cancer stem cells as well as the "quiescent" cancer stem cell pool for therapy.
We will analyze how these new concepts emerged from our analysis and systematic deconstruction of the molecular genetics of human cancer as studied in vivo in faithful mouse models, with particular emphasis on the role of aberrant PTEN/PI3K/AKT/mTOR signaling in tumorigenesis.

L.2

PROTEOMICS IN PATHOGENESIS AND DIAGNOSIS OF SYSTEMIC AMYLOIDOSES

Giampaolo Merlini
Department of Biochemistry, University of Pavia, Amyloidosis Research and Treatment Center, Biotechnology Research Laboratories, Fondazione IRCCS Policlinico San Matteo, Pavia – Italy

Protein misfolding and deposition as amyloid, with consequent tissue damage, play a key role in the group of diseases generically termed amyloidoses. In the systemic forms, amyloid deposition is widespread and causes severe dysfunction of vital organs. Proteomic analysis, thanks to its versatility and completeness of information, is an ideal tool for investigating systemic amyloidoses. Two-dimensional polyacrylamide electrophoresis (2D-PAGE)- and mass spectrometry (MS)-based proteomics is a useful approach to identify and characterize protein deposits in amyloid affected tissues, which is essential for designing the therapeutic strategy (Lavatelli et al. MCP 2008;7:1570-1583). Furthermore, proteomic application of laser dissection LC-MS/MS enable specific subtyping of inherited and AL-type amyloidosis. The proteomic analysis of tissues infiltrated by amyloid may shed light on the mechanisms of amyloid formation and of tissue targeting and damage. Shotgun proteomics, based on two dimensional liquid chromatography – tandem mass spectrometry (2DC-MS/MS) was applied to profile the proteome of abdominal subcutaneous fat tissue aspirates in patients with systemic amyloidoses and controls, and to perform label-free differential analysis (Mauri et al, Amyloid 2010;17 Suppl.1:55). Hierarchical clustering and principal component analysis of protein lists showed that patients and volunteers are grouped in distinct clusters, and data are contributing to the elucidation of the mechanisms of disease. Proteomics is now an essential tool in diagnosis and for gaining insights into the pathogenesis of systemic amyloidoses.

L.3

"QUANTITATIVE" PROTEOMICS FOR BIOMARKERS DISCOVERY AND VALIDATION

Sthephen Pennington
University College Dublin - CONWAY Institute of Biomolecular and Biomedical Research - Ireland

The application of a wide range of proteomics methods to the discovery of new protein biomarkers should serve to identify early markers of disease as well as predict and monitor patient response to therapeutic intervention. Together, these will inform decision making in the most effective use of existing drugs and therapeutic regimes and the development of new effective therapeutic strategies. In addition, the continued development of protein biomarkers to predict and monitor drug toxicity is likely to have a major impact on the drug development process. It is increasingly apparent that the validation of protein biomarker expression and qualification of utility is of critical importance and remains challenging.

This presentation will include

- examples biomarker discovery and validation in a range of diseases including pancreatic and prostate cancer
- the development of an approach for simultaneous quantitative measurement of the expression of cytochrome P450 isoforms to support predictive toxicology
- considerations for quantitative measurements of protein biomarkers by multiple reaction monitoring.

L.4

PHOSPHORYLATION EVENTS IN PLANT-PATHOGEN INTERACTIONS

Alex Jones
The Sainsbury Laboratory, Norwich – UK

Phosphorylation is probably the most highly studied of protein post-translational modifications and plays a central role in many aspects of cell biology including regulation of cell cycle, metabolism and defense responses. Although plants lack an adaptive immune system, they have an expanded family of protein kinases including a great diversity of receptor-like kinases (RLK), where extracellular domains such as leucine rich repeats and LysM motifs are frequently joined to an intracellular kinase domains. Such RLKs provide the first line of defense against pathogens by recognizing conserved elements such as flagellin and chitin thereby triggering signaling cascades to initiate defense mechanisms. Plant pathogens seek to modify defense responses and metabolism through the actions of secreted effector proteins. Such effectors - or their actions - may in turn be recognized by the host cell leading to further defense responses, typically localized cell death.
I will discuss the identification and quantification of differential phosphorylation events at several stages of defence pathways, taking examples from bacterial, fungal and oomycete pathogens. I will present new data on plant receptor-like kinases (CERK1 and BAK1), an intracellular kinase involved in resistance signalling (Pto) and a secreted effector protein kinase (CRN8) from Phytophthora infestans.

L.5

THE SPECIFICITY OF MASS SPECTROMETRY IN BIOMOLECULAR RESEARCH. PRESENT AND FUTURE INNOVATION IN NATURAL AND HEALTH SCIENCES

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The specificity of mass spectrometry (MS) in the biomolecular sciences[1] is a touch of innovation in present and future applications of the methodology in natural and health sciences. Weighing and identifying ionic species in complex mixtures is a formidable task that MS does since ever and that can be exploited for molecules ranging from two to million Daltons. The last century '80s have seen the flourishing of spray and desorption methods allowing *elephants* to fly and recognized through the reconstruction from their specific fragments. This classical approach to structure determination is still used when the complexity of a biopolymer is firstly reduced, i.e. by catalysis, and than rebuilt as a puzzle game.

The high resolution and the high mass range of modern mass spectrometers allow also the detection of intact large molecules, whereas innovation in both ionization and detection method, enormously improved in the last few years, allows the stepwise analysis of an ion family, in-time or in space, with a high degree of accuracy and reliability. The way therefore for any exploitation dealing with molecular structure has been paved by the pioneers of the methodology.

The role assumed by desorption methods hyphenated with MS/MS facilities in proteomics, lipidomics, genomics, transcriptomics and the like, calls also for the development of molecular devices and new analytical procedures to reduce substantially the complexity of the matrixes to be examined[2].

References:

[1]*Mass Spectrometry in Biomolecular Sciences* .R. M. Caprioli A. Malorni and G. Sindona eds. Kluwer Academic, Dordrecht, 1996.

[2]Exploitation of Endogenous Protease Activity in Raw Mastitic Milk by MALDI-TOF/TOF.. A. Napoli, D. Aiello, L. Di Donna, H. Prendushi, G. Sindona. Anal. Chem. 2007, 79, 5941-5948.

L.6

TRANSITION FROM GENOMICS TO A POST-GENOMIC ERA IN MEDICALLY RELEVANT FUNGI: APPLICATION OF PROTEOMICS IN CLINICAL DIAGNOSIS AND BIOMARKER DISCOVERY

Maurizio Sanguinetti
Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Largo F. Vito, 1 00168 Rome, Italy

The completed genomes of a variety of clinically relevant fungal species, most of them belonging to the genera *Aspergillus* and *Candida*, has recently highlighted the necessity to shift toward a post-genomic research, since knowledge of the expression levels of gene products has become necessary to fully utilize the wealth of information provided by large-scale genome-sequencing projects. Moreover, it has been shown that gene networks cannot be completely understood without information about protein expression. Among the many 'omic' techniques available, proteomics are of particular interest, thus representing the more direct studies of cellular functions at the protein level. By comparing the proteomic signature of the reference state to a proteomic signature derived under experimental conditions (e.g., biofilm growth or drug exposure) or from a clinical setting (e.g., invasive infection), it has been possible to obtain important insights about the biology of a fungal pathogen.

Recently, the application of proteomic tools gave a comprehensive overview about the proteins of *Aspergillus fumigatus* present or induced during environmental changes and stress conditions. Detecting some of the gene products expressed under stress conditions are also known fungal antigens, such as the thioredoxin peroxidase AspF3, proteomics offer the potential to screen for new antigens that could improve the diagnosis of diseases caused by *A. fumigatus*. Furthermore, by using immunoproteomics (i.e., 2D gel electrophoresis followed by Western blotting), a repertoire of novel *Candida albicans* antigens have been identified, that elicited a specific immune response in systemic candidiasis patients undergoing malignant haematological disorders. Remarkably, these studies also showed that differences in antigen recognition patterns correlated with infection progression, thereby providing new challenges for diagnosis and clinical follow-up of *C. albicans* infections.

To summarize, different proteomic approaches in the field of medical mycology, while are contributing to a deeper and time-dynamic understanding of the fungal biology, are becoming effective for the discovery of new biomarkers to be used not only for the diagnosis of fungal infections but also for specific therapeutic interventions, and ultimately for the discovery of novel vaccine candidates.

L.7

PROTEOMICS AND GENOMICS

Joachim Klose
Charité *Campus* Virchow-Klinikum Berlin, Germany

ABSTRACT NOT AVAILABLE

ABSTRACTS

1.1

DISTINCTIVE MOLECULAR SIGNATURES IN MULTIPLE SCLEROSIS: PROTEOMIC AND LIPIDOMIC INVESTIGATIONS

Piero Del Boccio[a,b], Damiana Pieragostino[a,b],Viviana Greco[c,d], LuisaPieroni[c,d], Maria Di Ioia[e], Alessandra Lugaresi[b,e], Domenico Gambi[b,e], Marco Onofrj[b,e], Giorgio Federici[c;d] Carmine Di Ilio[a,b], Paolo Sacchetta[a,b], Andrea Urbani[c;d].

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Multiple Sclerosis (MS) is defined as a chronic inflammatory and demyelinating disease that affects the central nervous system. From more recent studies it has been brought forward the hypothesis that MS is a secondary event to an autoimmune response against myelin components to self antigens in a genetically susceptible host, which leads to a chronic and progressively debilitating disease. Nowadays MS diagnosis is based both on a set of objective analysis (as the study of oligoclonal bands or Nuclear Magnetic Resonance), which requires that signs and symptoms are evident. Some of these symptoms are common to other neurodegenerative and psychiatric disorders, so that interpretation of these signs often becomes very difficult, despite the detailed guidelines used. We have developed and employed molecular open-platform strategies for the identification and characterization of proteins and metabolic alterations in biological samples induced by onset of MS. This approach aimed to investigate the effects of the disease on protein/metabolic profiling, possibly addressing the multiple factors characterising MS. In particular we focused our attention on investigating serum, plasma and CSF protein profiling and polar lipids patterns in MS patients, healthy controls and subjects with other inflammatory neurological diseases. Results showed changes in proteome/lipidome of MS patients opened the route for the discovery of novel candidate biomarkers of disease onset and progression. A tentative identification of the differential bio-molecules highlighted was carried out, give us the opportunity of correlate specific protein-lipid profiles as definite functional molecular biomarkers.

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1.2

LIPIDOMIC APPROACH TO RCC PATIENTS URINARY EXOSOMES FOR THE DETECTION OF POTENTIAL TUMOR MARKERS

Piero Del Boccio [a,b], Francesca Raimondo[c], Damiana Pieragostino [a,b], Lavinia Morosi[c], Francesco Rocco[d], Paolo Brambilla[c], Luca Caruso[e], Andrea Urbani[b], Paolo Sacchetta[a,b], Fulvio Magni[c], Marina Pitto[c]

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[e]Dept. of Medicine Surgery and Dentistry, Pathological Anatomy, S. Paolo Hospital, Milano, Italy

Urinary exosomes, derived from every epithelial cell type facing the urinary space, have been proposed as starting material for discovery of molecular biomarkers of kidney disease. Clear cell renal carcinomas (RCC) is representing about 3% of all kidney cancers. No biomarkers for early diagnosis of RCC are yet available. Here we addressed the lipidomic analysis of the exosomes isolated from control and RCC patient urines in order to look for differences to be exploited as potential tumor marker.
Extracted lipids were analyzed using a CapLC system coupled on-line with a Q-TOF-MS instrument equipped with a nano-Lock-Spray source. Elution was obtained using C18 column with a water/tetrahydrofuran gradient. The method was developed and optimized using six representative lipids and deuterated sphingomielin as internal standard. Centroid data acquisition in the 300-2000 m/z range and GluFibrinoPeptide as reference compound for on-line recalibration data were used.
Results show differential lipid pattern of RCC samples in respect to control specimens. Structural characterization of differential lipids highlighted was tentatively performed through the following analytical strategies: fragmentation experiments by mass spectrometry and lipid data bank search for accurate mass matching. In conclusion our work suggests that lipidomics of urinary exosomes show potential for the identification of RCC biomarkers.Validation of this results will be necessary to confirm the potential biomarkers highlighted, in order to give an important functional insight on their role and implication in the pathological process.
The present work has been supported by FIRB: Rete Nazionale per lo studio del proteoma umano (n. RBRN07BMCT).

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1.3

IDENTIFICATION OF DIFFERENTLY EXPRESSED ION SIGNALS BY MALDI-TOF MS PROFILING IN SUBSETS OF MOLECULARLY DEFINED BINET STAGE A CHRONIC LYMPHOCYTIC LEUKEMIA(CLL).

Sabrina Bossio[1,2], Elena Urso[1], Maria Le Pera[1], Teresa Sprovieri[1], Anna Grazia Recchia[2], Fortunato Morabito[2] and Antonio Qualtieri[1]

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2. U.O.C of Ematology, Annunziata Hospital of Cosenza, Cosenza 87100, Italy

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the western world characterized by a variable clinical course. MALDI-TOF MS profiling was utilized to examine the proteome complexity of 42 untreated newly diagnosed Binet stage A CLL patients in. Highly purified B lymphocytes were subjected to cell lysis, followed by sub-fractionation including a cytoplasmic, water-soluble component whose peptide/protein profiles were analysed using a VoyagerDe PRO MALDI-TOF mass spectrometry (PerSeptiveBiosystem).Separate spectra were obtained for a restrictive mass-to charge (m/z) range (1000-25000Da) in linear geometry, using an accelerated voltage of 25kV. The acquired spectra, in duplicate, were processed for automated advanced baseline correction and noise. The ion peak area signal was normalized as a percentage of the total peak area (individual peak area/total area per cent). Supervised analysis for CD38, ZAP-70 and IgVH mutational status was conducted revealling23, 32 and 28 statistically significant differentially expressed ion signals ($p<0.05$, Student's t-test), respectively in CD38 (14 positive), ZAP-70 (13 positive) and IgVH (12 unmutated). Six m/z values (2162.9; 2782,5; 3110,5; 4572,3; 6973,6; 10463,2 m/z values) were shared by the three peak lists. Supervised analysis conducted using a prognostic score system that compared cases scored 3 (i.e. CD38-positive, ZAP-70-positive and unmutated-IgVH) with those cases scored 0 (i.e. with no negative prognostic marker) identified 25 statistically significant differentially expressed ion signals ($p<0.05$), among which were recognized the 6 ion signals mentioned above. These results demonstrate that the CLL neoplastic cells may be classified on the basis of their protein/peptide content using MALDI-TOF profiling analysis.

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1.4

HUMAN TEARS PROTEOMIC INVESTIGATIONS ON PSEUDOEXFOLIATION SYNDROME (PEX)

Damiana Pieragostino[a,b], Luca Agnifili[c], Vincenzo Fasanella[c], Sonia Bucci[a,b], Antonio Di Muzio[a,b], Marco Ciancaglini[d], Leonardo Mastropasqua[c], Andrea Urbani[b,e], Carmine Di Ilio[a,b], Paolo Sacchetta[a,b] and Piero Del Boccio[a,b]

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Pseudoexfoliation (PEX) syndrome is a generalized disease of the extracellular matrix and the major cause of severe open-angle glaucoma. Extracellular PEX material is produced, aggregated and accumulated in the anterior segment of the eye. Ocular depositions of PEX material can lead to many intraocular complication. Unfortunately PEX material composition is still unknown. Ocular tear film is a thin layer of fluid that covers the ocular surface and the study of tear proteome may give useful information for the understanding of physio-pathological mechanisms underlining to PEX syndrome. This biological fluid is not studied enough because the low quantity produced. We developed a new method for tear proteins extraction and analysis in order to highlight candidates biomarker of PEX. By the use of different proteomic methods, such as monodimensional electrophoresis and mass spectrometry technologies we characterized PEX tear protein profiling. Differentially expressed protein were identified through ESI-MS/MS fragmentation analysis. Our findings showed differentially expressed proteins and particularly high level of immunoglobulins in tears of patients with PEX and the absence of these proteins in healthy control subjects and glaucoma patients. These effect was never described before in this biological compartment. Thus our data could help to describe the phenomena related to PEX material production and composition, in a biological fluid very easy to obtain. The presence of inflammation-associated proteins in tears of PEX subjects may reflect the inflammatory conditions of PEX disease, a systemic disorder that generate first symptoms and damages in the eye.

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1.5

HUMAN PLASMA PROTEOME OF PATIENTS WITH ABDOMINAL AORTIC ANEURYSM

Tania Gamberi [a], Michele Puglia [b], Francesca Magherini [a], Francesca Guidi [a], Luca Bini [b], Pietro Amedeo Modesti [c] and Alessandra Modesti [a]

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Abdominal aortic aneurysm (AAA) is an important health problem. In cross-sectional population studies of older men the prevalence varies from 3% to 8%. AAA causes about 1% of deaths in developed countries. In elderly men AAA may cause as many as 2% of all deaths [1]. The incidence of asymptomatic and ruptured AAA has increased in several countries during the last few decades. Consequently, an early diagnosis is desirable and the identification of circulating biomarkers may to this aim. Plasma proteins are useful targets for diagnostic, prognostic, and therapeutic development. With proteomic tools available, profiling of human plasma proteome becomes more feasible in searching for disease-related markers [2]. The aim of this study was to identify biomarkers of abdominal aortic aneurysm using 2DE and mass spectrometry. We investigated the plasma proteome from 8 patients with abdominal aortic aneurysm. The control group included 6 age-matched volunteers. Whole blood was collected into K2EDTA-containing tubes and analysed by 2DE using a pH range of 3 to 10. We identified 53 spots whose density significantly changed in patients with AAA in comparison with the control group. Most of the proteins identified are acute phase proteins such as haptoglobin, α-2-HS glycoprotein, α-2 antichimotrypsin, ceruloplasmin, α-2 antiplasmin and they are significantly increased in AAA group when compared with control subjects. Moreover, in patients we observed the increase of three isoforms of fibrinogen gamma chain and the increase of the immunoglobulin gamma chains.

The present work is merely an example of the use of proteomics to identify potential biomarkers from patients with AAA in plasma.

This work was supported by the FIRB project "Italian Human ProteomeNet" (BRN07BMCT_013), from MIUR.

References:

[1] Schermerhorn ML, Cronenwett JL. (2001) *J Vasc Surg*. 33, 443.

[2] Anderson, N. L. & Anderson, N. G. (2002) *Mol. Cell. Proteomics* 1, 845–867.

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1.6

A LABEL-FREE QUANTITATIVE APPLICATION OF MALDI–TOF–MS TO MEASURE THYMOSIN BETA 4 LEVELS IN HUMAN CEREBROSPINAL FLUID

Elena Urso, Maria Le Pera, Sabrina Bossio, Teresa Sprovieri and Antonio Qualtieri

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been applied to the analysis of a wide range of biomolecules. To date the application of MALDI-TOF-MS is viewed as impractical for the analysis of low-mass analytes and for relative quantitative applications. However, these limitations can be overcome and quantification can be routine. Increased levels of thymosin beta (TB4) have been recently found in cerebrospinal fluid (CSF) from Creutzfeldt-Jakob disease (CJD) patients. Our objective was to measure TB4 levels in human CSF applying a MALDI-TOF-MS label-free quantitative method based on the TB4 oxidized form, as an internal standard . The quantification of TB4 was carried out on CSF samples from a group of 10 healthy subjects examined for idiopathic intracranial hypertension and shown to be neurologically normal without radiological abnormalities on magnetic resonance imaging and in a group of CSF samples derived from 6 patients with CJD. The relative peak area or peak height ratios between native TB4 and the added TB4 oxidized form were evaluated. The values of TB4 in CJD patients were found higher than controls, as previously reported. The proposed method may provide a simple and rapid screening for quantification of TB4 levels in CSF, suitable for diagnostic purposes.

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1.7

PROTEOMIC APPROACH TO IDENTIFY PI3K/AKT DOWNSTREAM TARGETS

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Phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway is involved in the regulation of cell proliferation, apoptosis, migration, angiogenesis and inflammatory responses in several cell types. The Akt kinase family is composed of three highly homologous isoforms: Akt1 (PKBalpha), Akt2 (PKBbeta) and Akt3 (PKBgamma). Analysis of the Akt isoforms has established that the function of the different kinases is not completely overlapping and that isoform-specific signaling contributes to the diversity of Akt activities.

In this study, lentivirus-mediated small interfering RNA was employed in lung adenocarcinoma cell line H460 to downregulate endogenous AKT1/AKT2/PI3K expression with the aim to investigate the role of PI3K/AKT signaling pathway, which is frequently hyperactive in a number of human diseases and plays a key role in the development of many human cancers.

Two-dimensional gel electrophoresis technology was used to perform protein profiling and to define PI3K/AKT downstream targets. Proteins differentially expressed were analyzed by software analysis (Image Master 2D platinum GE), subjected to in-gel digestion, identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and further verified by western blot analysis. Several proteins, up or down-regulated after SiRNA interference, were identified, most of which are involved in proliferation, adhesion and tumorigenesis pathways.

Some examples of changes in protein expression include: Rho GDP-dissociation inhibitor 1 implicated in signal transduction, Prohibitin and Proliferating cell nuclear antigen involved in cell proliferation.

The results presented here, even though preliminary, might contribute to the understanding of the functional specificity of the Akt isoforms and to elucidate several aspects concerning the involvement of PI3K/AKT pathway in human diseases such as cancer.

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DOPAMINE STIMULATION OF JURKAT HUMAN CD4+ T-CELL LEUKEMIA CELLS. A PROTEOMIC ANALYSIS.

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The functions of immune cells are regulated not only by cytokines, but also by several neurotransmitters. In particular, peripheral blood lymphocytes possess a complex dopaminergic regulatory system [1]. Human T-lymphocytes express all five dopamine receptors (D1-D5), each of which exerts different actions on the regulation of T-cell functions [1]. The Jurkat human CD4+ T-cell leukemia cell line has been thoroughly used and characterized as a suitable cell model to investigate T-cell signalling and apoptosis [2], nevertheless its characterization as a model of circulating dopaminergic cells was never reported before. In this regard, we characterized the dopaminergic system in Jurkat cells and through a proteomic approach we analyzed their response to a dopamine challenge in order to highlight metabolic pathways that could specifically reveal alterations linked to PD. A dopamine challenge, with no effect on cell viability (50 μM), induced quantitative changes in the protein expression pattern. The proteins that showed quantitative differences were identified by peptide mass fingerprinting and analyzed in terms of both interaction network and GO classification enrichment using *PPI spider* [3]. We obtained a significant model for network enrichment based on known protein-protein physical interactions and a functional association to Gene Ontology (GO) classification GO:0051082 (unfolded protein binding).

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1.9

IRON METABOLISM IN MURINE MACROPHAGES: A PROTEOMIC INSIGHT.

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Macrophages play a critical role at the crossroad between iron and immunity, being able to store and recycle iron derived from the phagocytosis of senescent erythrocytes. The way by which macrophages manage non-heme iron at physiological concentration is still not fully understood. We performed a 2-DE differential analysis to investigate the protein changes in Mouse Bone Marrow macrophages incubated with ferric ammonium citrate (FAC 10nM) for 24h in serum-free media.

Differentially expressed spots were identified by nanoRP-HPLC-ESI-MS/MS. FAC induced the over-expression of ferritins, a family of iron storage proteins, and complex variations of several proteins of the cytoskeleton, which has been implicated in cytoplasmic distribution of ferritin molecules. Of note, FAC up-regulated the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (↑ 2.36) at the membrane level, where it has been recently proposed to act as an alternative transferrin receptor for iron acquisition through internalization of the GAPDH-transferrin complex into the early endosomes. Among membrane associated proteins, FAC also up-regulated vimentin (↑ 4.54) and galectin-3 (↑ 1.97), both known to be involved in phagocytosis/internalization processes. Among cytosolic proteins, FAC induced de novo expression of macrophage inhibitory factor (MIF), a cytokine that plays a critical role in the inflammatory response. This proteomic study represents an example of the potential role of this methodology in the field of iron biology, especially for the elucidation of the molecular mechanisms controlling iron homeostasis in normal and disease conditions.

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2D IMMUNOMIC APPROACH FOR THE STUDY OF IgG AUTOANTIBODIES IN THE EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS

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The identification of autoantigens recognized by autoantibodies has provided no conclusive results in most neurological autoimmune diseases. Bi-dimensional immunomics may represents a useful tool in this regard, but its application may be limited by the denaturing condition of target proteins. We compared the capacity of autoantibodies in sera of mice affected by experimental autoimmune encephalomyelitis (EAE) to recognize neural autoantigens by two different assays, ELISA and 2D immunomics. We induced the disease with peptide$_{89\text{-}104}$ of the myelin basic protein (MBP), total MBP or spinal cord homogenate (SCH), to verify whether the complexity of autoantigen quantitatively influenced the autoimmune response. Concordant results have been obtained with both techniques, which showed the presence of anti-MBP IgG only in mice immunized with total MBP. Apart from MBP, 2D immunomics revealed the presence in all EAE mice of seric autoantibodies targeting other neural proteins, such as myelin oligodendrocyte glycoprotein and 2', 3'-cyclic nucleotide 3'-phosphodiesterase. The presence of epitopes with partial sequence homology among these proteins and MBP may suggest a process of inter-molecular mimicry. These findings by 2D immunomics of multiple neural proteins targeted by autoantibodies generated by a single antigen may help to explain the complex autoimmune response observed in multiple sclerosis.

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PLASMA PROTEINS CARBONYLATION AND PHYSICAL EXERCISE

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Regular physical activity is associated with a reduced risk of coronary heart disease, probably modifying the balance between free-radical generation and antioxidant activity. On the hand, acute physical activity increases oxygen uptake and free-radical production. An increase in oxygen consumption leads to a temporary imbalance between the production of RONS and their disposal; this phenomenon is called oxidative stress. Proteins are one of the most important targets of oxidation during physical exercise and carbonylation is one of the more common oxidative protein modifications(1). An increase in oxidized protein levels in cells may cause a series of cellular malfunctions that could lead to a disease state. For this reason a quantification of protein oxidation is of major importance to distinguish healthy and disease states. The aim of our research was to characterize plasma proteins carbonylated in response to physical exercise (PE) in men trained to perform endurance exercise. It is widely accepted the increase of carbonylated proteins in plasma of athletes after exercise, but in literature there aren't works in which the targets of this oxidation are identified. We analyzed the plasma taken at resting condition and after two different kinds of physical exercise (a power test and a resistance test) by a proteomic approach using 2D-GE followed by western blot with specific antibodies against marked carbonylated proteins. From the 2D analysis we found proteins target of carbonylation after physical exercise, but also proteins which carbonylation is not affected by exercise and proteins carbonylated only at resting condition. These methods have allowed to obtain an overview of the change in the oxidation of plasma proteins after physical exercise and to identify new markers of physiological stress.

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2DE-WB ANALYSIS OF A ISOFORM OF RAT STEROID 5 ALPHA REDUCTASE

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The enzyme 5a-reductase (5a-R) is present in many mammalian tissues, including the brain, and exists as two isoforms, 5a-R type 1 (5a-R1) and 5a-R type 2 (5a-R2). The physiological importance of 5a-R in the brain may derive from two of its properties: its capability to convert testosterone (T) to a more potent androgen, dihydrotestosterone (DHT); and its capability to convert progesterone and deoxycorticosterone (DOC) to their respective 5a-reduced derivatives. For this reason, both isozymes of 5a-R may play an important role in the physiology and pathology of the CNS not only during ontogenesis, but also throughout the individual's life. The two isoforms have a theoretically pI of 9.40 and are membrane proteins with a molecular weight of about 29 KDa. Two dimensional data are not available on the presence and distribution of the 5a-R isoforms in the rat brain probably due to low expression and to extreme physical properties. In the present study, experiments were conducted in triplicate using different pH ranges and different solubilisation solutions to optimize spot resolution and to visualize different isoforms of 5a-R on blots. Quantitative 1D and 2D western blotting with homemade antibodies were performed on two brain areas (medial prefrontal cortex and nucleus accumbens) of rats. Image analysis were performed with Quantity One (Biorad) and Progenesis SameSpots software (Nonlinear Dynamics). Image analysis of 1D western blot showed a sharp band at about 20 KDa according to literature data and a sharp spot at the same MW but at acidic pI. This unexpected finding is now under validation by mass spectrometry.

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1.13

IN SILICO ANALYSES OF PROTEOMIC DATA SUGGEST A ROLE FOR HEAT SHOCK PROTEINS IN UMBILICAL CORD BLOOD HEMATOPOIETIC STEM CELLS

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Umbilical-cord blood (UCB) has growingly become an accepted alternative source of hematopoietic stem cells for transplantation purposes. However, the low cell dose limit within a single unit is still an obstacle hindering the way of a broader diffusion. The real deal is the lack of knowledge about the molecular processes governing the events of expansion and differentiation of these cells. In order to fill this void, several studies were focused on the identification of the peculiar whole protein profile of UCB-derived hematopoietic stem cells. In this review article we provide a referenced list of overall proteins from UCB-derived hematopoietic stem and progenitor cells. This list has been elaborated for pathway and network analyses, along with GO term enrichment for biological and molecular functions, in order to individuate main classes of proteins governing functioning of these cells. From these analyses it seems to emerge a central role for heat shock proteins in immature hematopoietic stem cells. Their role might be relevant in protecting crucial transcription factors which drive proliferation and differentiation towards a specific lineage (e.g. erythroid, myeloid). Hereby we also stress the helpfulness of interactomics elaboration in providing a unified overview of independent proteomics data. It appears that maturation, other than representing a bottleneck to protein expression, could sculpt interaction maps via reducing complexity of immature interactomics profiles.

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THE RED BLOOD CELL PROTEOME AND INTERACTOME: AN UPDATE

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Although a preliminary portrait of the red blood cell proteome and interactome has been already provided, the recent identification of 1,578 gene products from the erythrocyte cytosol asks for an updated and improved view. In this paper we exploit data available from recent literature to compile a non-redundant list of 1,989 proteins and elaborate it with pathway and network analyses.
Upon network analysis it is intuitively confirmed that red blood cells likely suffer of exacerbated oxidative stress and continuously strive against protein and cytoskeletal damage. It also emerges that erythrocyte interaction networks display a high degree of maturity. Indeed, a series of core proteins were individuated to play a central role. A catalytic ring of proteins counteracting oxidative stress was individuated as well. In parallel, pathway analysis confirmed the validity of observations about the SEC23B gene role in CDA II in a fast and unbiased way.

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USE OF PROTEOMINER™ TECHNOLOGY FOR THE DETECTION OF LOW ABUNDANT PROTEINS IN PLATELET SECRETOME DURING 7 DAYS OF STORAGE

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The limited ability to disclose the so called "hidden proteome" [1] represents one of the major limits of modern proteomics. This is particularly true in the analysis of body fluids, considering the vast dynamic range [2] of the proteins present therein and the great preponderance of albumin, which alone accounts for the 60% of protein amount. Proteomic strategies aimed at increasing the visibility of low abundant proteins are continuously tweaked, among which the equalization technology commercially known as ProteoMiner. In the present study, ProteoMiner has been used to analyse the supernatant of gas-permeable storage bags of platelet concentrates (PCs) from buffy coats suspended in plasma 100% after 0, 4 and 7 days of storage at 22°C. The use of ProteoMiner increased the number of detectable spots with respect to non-equalized counterpart sampled at the same day of storage (329±9 spots vs 304±7 at day 0, 326±10 vs 305±9 at day 4, 331±8 vs 308±9 at day 7). Further MS analysis revealed several platelet-derived proteins that were detectable only in the analysis of equalized samples, such as platelet beta tropomyosin and vitronectin. Nonetheless, platelet-derived proteins have been also revealed in 2D maps of non equalized samples (platelet alpha-1-B-glycoprotein, high molecular weight kininogen and proteinase inhibitor 8). These results showed that ProteoMiner could be a valuable tool in dissecting platelet secretome during storage.

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STANDARDIZED SAMPLE PREPARATION PHASES FOR A QUANTITATIVE MEASUREMENT OF PLASMA PEPTIDOME PROFILING BY MALDI-TOF

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MALDI-TOF MS is a powerful technique for studying plasma and serum, thus enabling the detection of proteins, and the generation of reproducible protein profile mass spectra, potentially able to discriminate correctly different biological systems. An assay has been developed for the detection of low-abundant and low molecular weight proteins, from human plasma, aiming at the identification of new potential biomarkers.
The high molecular weight proteins were cut off by Centricon and the plasma peptidome was cleaned by Zip Tip C18. Spectra were acquired by Voyager-DE STR and aligned by the software ALISPECTRA.
The different steps of the pre-analytical phase that may affect the reproducibility of plasma proteome analysis have been carefully considered. The results showed that the method is highly accurate (9.1%) and precise (8.9%) and the calibration curve for the ACTH (18–39), in human plasma, gave a good correlation coefficient ($r>0.99$ and $r^2>0.98$). The limit of detection (LOD) and the limit of quantification (LOQ), with relative intensity experimentally calculated, were of 0.5×10^{-9} M and $1.0\times10-9$ M in that order.
The method was tested on plasma from patients with a first diagnosis of pelvic mass. The peaks were used as variables for the statistical analysis: Analysis of variance (ANOVA) and Principal Component Analysis (PCA) were performed. Statistical analysis of plasma profile generated a sub-profile of 17 peptides with their relative abundance able to discriminate patients bearing malignant or benign tumors. The sensitivity and specificity were 84.7% and 80.0% respectively.

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ON THE PRO-CALCIFYING SIGNATURE OF DERMAL FIBROBLASTS: NEW INSIGHTS FROM THE PXE MODEL

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Inappropriate biomineralization of soft connective tissues (SCT) may occur as a complication of several disorders and with aging. Within SCT, elastic fibers are the most susceptible to calcification, being constituted by a polymer with a very low turnover. Calcifications are at the basis of impaired elastic fiber deformability upon mechanical stretching, mainly affecting cardiovascular system, lungs, dermis and Bruch's membrane.

To shed light on the pathogenetic mechanisms responsible for pathologic calcification of SCT, a very interesting model is represented by Pseudoxanthoma elasticum (PXE), a genetic connective tissue disease characterized by progressive mineralization of elastic fibres. We have investigated if PXE fibroblasts have a bone-oriented phenotype, as shown for smooth muscle cells during blood vessel calcification. Data indicate that PXE cells do not show any shift towards an osteoblast-like phenotype, since expression of genes encoding for bone markers, as ectonucleotide pyrophosphatase/phosphodiesterase family member 1, progressive ankylosis protein homolog, carbonic anhydrase II, bone morphogenetic protein 2 and 7, was similar to controls. Interestingly, PXE fibroblasts exhibited a significant up-regulation of tissue non-specific alkaline phosphatase (ALPL/TNAP), as revealed by RT-PCR, WB and enzyme activity assay. Interestingly, alkaline phosphatase activity in patients' serum was always within laboratory reference values. Furthermore, PXE fibroblasts exhibited modified expression of some osteopontin (OPN) isoforms. This study represents the first evidence in PXE fibroblasts for a local abnormal production and activity of TNAP and for an altered balance between posttranslationally modified OPN isoforms which may influence mineralization in PXE soft connective tissues, thus opening new perspectives for therapeutic approaches.

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PROTEOMIC ANALYSIS OF PLASMA FROM PATIENTS UNDERGOING CORONARY ARTERY BYPASS GRAFTING REVEALS A PROTEASE/ANTI-PROTEASE IMBALANCE IN FAVOR OF THE SERPIN α1-ANTICHYMOTRYPSIN

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We used proteomics to identify systematic changes in the plasma proteins of patients undergoing coronary artery bypass grafting (CABG) by means of cardiopulmonary bypass surgery. It is known that, after CABG, a complex systemic inflammatory responses ensues that favours the occurrence of adverse postoperative complications frequently recognizing inflammation itself and/or thrombosis as the underlying mechanism.

We found a marked and persistent postoperative increase in the levels of the serin-protease inhibitor α_1-antichymotrypsin (α_1-ACT) that fully maintains the inhibitory activity blunting its protease substrate cathepsin G. An intraoperative increase followed by a rapid decline in proteases activation was documented, accompanied by a substantial induction of leucine-rich-α-2-glycoprotein, a protein involved in neutrophilic granulocyte differentiation. Finally, a time-dependent alteration in the expression of haptoglobin, transthyretin, clusterin and apoE was observed.

In conclusion, we showed that after CABG, a protease/antiprotease imbalance occurs with early cathepsin G activation and a more delayed increase in α_1-ACT. As cathepsin G is a serpin involved both in inflammation and coagulation activation, this confirms and expands the concept of a marked dysregulation of both inflammatory and hemostatic balances occurring after CABG. And the pharmacologic modulation of this imbalance may be a new therapeutic target to reduce postoperative complications.

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THE SECRETOME OF ENDOTHELIAL CELL: A TOOL FOR THE STUDY OF THE PLEIOTROPIC EFFECTS OF STATINS

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The clinical benefits of 3-hydroxy-3-methyl-3-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are strongly related to their low density lipoprotein-cholesterol lowering properties. However, because mevalonic acid, the product of HMG-CoA reductase reaction, is the precursor not only of cholesterol but also of nonsteroidal isoprenoid compounds, the inhibition of HMG-CoA reductase may result in pleiotropic effects. Indeed, a variety of experimental data indicates that statins can interfere with major events involved in the formation of atherosclerotic lesions, independent of their hypocholesterolemic properties, although these effects have not been fully elucidated. In this respect, the application of a global proteomic approach to determine the effect of statins on the proteins released, "secretome", by endothelial cells could help to understand novel mechanisms by which statins promote some of their beneficial effects. Two methods were applied to the identification and quantification of proteins differentially regulated by statins: a "gel based method" employing 2-DE, which can offer the additional advantage to distinguish between proteins isoforms as well as different post-translationally modified forms of the same proteins, and a "gel-free MS-based method" for "label-free" quantitation, which provided absolute quantitative profiling of proteins. The results coming from the application of both approaches were integrated, validated by biochemical assays, and allowed us to fully characterize the secretome of endothelial cells and to identify the drug-regulated proteins. In conclusion, secretomes are a rich source of new therapeutics and drug targets, and have the potential to become a major focus of drug discovery programs throughout the industry.

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CHANGES OF NUCLEAR PROTEOME IN RESPONSE TO DOWN-MODULATION OF Vav1 DURING MATURATION OF TUMORAL MYELOID PRECURSORS

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Vav1 is physiologically expressed only in hematopoietic cells and its presence in other tissues is constantly associated with the appearance of malignant features. Even though in all cell models the best known role of Vav1 is a tyrosine phosphorylation-regulated GEF activity, a number of different functions has been recently attributed to this protein, including its involvement in gene transcription. Vav1 is a key molecule in the overcoming of the differentiation blockade of tumoral myeloid precursors, in which it actively sustains the agonist-induced acquisition of a mature phenotype [1]. In particular, by means of a proteomic approach, we have demonstrated that Vav1 plays a role in modulating the protein tool by means of which ATRA executes the maturation program of APL-derived cells [2], possibly as part of transcriptional complexes [3]. Since high amounts of Vav1 accumulate inside the nuclear compartment of ATRA-treated promyelocytes, the possible role of this protein in modulating the nuclear proteome was investigated. With this aim, nuclei purified from APL-derived cells induced to differentiate in the presence of forcedly down-modulated Vav1 were subjected to two dimensional differential in-gel electrophoresis (2D-DIGE) followed by mass spectra analysis. The nuclear amount of about 30 ATRA-induced proteins, including nucleoskeleton components, RNA-binding proteins, splicing factors and regulators of gene transcription, resulted significantly affected when Vav1 was down-modulated during the differentiation treatment. The involvement of Vav1 in the ATRA-dependent modulation of gene transcription was demonstrated for some of the identified proteins, like the SFRS1 splicing factor and the HNRNPK component of hnRNPs. Our results provide the first evidence that, at least in maturation of tumoral myeloid precursors, Vav1 is actively involved in modulating the complex nuclear network underlying protein expression. These findings may constitute the starting point for studies aimed to develop innovative strategies targeting Vav1, useful for the management of both leukemias and solid tumours expressing this protein.

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PROTEOMIC AND REDOX-PROTEOMIC ANALYSIS OF ALKAPTONURIC OCHRONOSIS IN EX VIVO CELL MODELS

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Alkaptonuria (AKU) is a rare genetic disease associated with the accumulation of homogentisic acid (HGA) [1] and its oxidized/polymerized products in connective tissues up to the deposition of melanin-like pigments (ochronosis) [2]. Although numerous case reports have described this phenomenon in articular cartilages, little is known on the molecular mechanisms leading to alkaptonuric ochronosis. For this reason, we undertook a proteomic and redox-proteomic analysis on osteoblasts, chondrocytes, and synoviocytes from an alkaptonuric patient undergone hip replacement.

Human articular cartilage/bone fragments were obtained after ethics approval and patient's informed consent. After enzymatic digestion, cells were cultivated in medium added with 0.33 mM HGA prior to analysis. Based on the macroscopic appearance of ochronotic cartilage, two sub-populations of chondrocytes were identified: cells coming from the black portion of the cartilage were referred to as "black" AKU chondrocytes, while those coming from the white portion were referred to as "white" AKU chondrocytes. 2D-PAGE and Western Blot analysis of carbonylated proteins were used to investigate the protein repertoires of AKU cells in comparison with healthy control cells.

Our results showed only minor changes in AKU osteoblasts, while AKU synoviocytes and "black" and "white" chondrocytes seemed to be more susceptible to the ochronosis-induced stress. In particular, profound alterations in the levels of proteins involved in cell defence, protein folding and cell organization were highlighted, together with extensive post-translational oxidation of proteins, which was particularly important in "black" AKU chondrocytes.

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1.22

PROTEOMIC AND REDOX-PROTEOMIC EVALUATION OF HOMOGENTISIC ACID AND ASCORBIC ACID EFFECTS ON HUMAN ARTICULAR CHONDROCYTES

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Alkaptonuria (AKU) is a rare genetic disease associated with the accumulation of homogentisic acid (HGA) [1] and its oxidized/polymerized products in connective tissues up to the deposition of melanin-like pigments (ochronosis) [2]. Since little is known on the effects of HGA and its metabolites on articular cells, we carried out a proteomic and redox-proteomic analysis to investigate how HGA and ascorbic acid (ASC) affect the human chondrocytic protein repertoire.

We settled up an *in vitro* model using a human chondrocytic cell line to evaluate the effects of 0.33 mM HGA, alone or combined with ASC. ASC was initially adopted to treat AKU in order to prevent the oxidation of HGA into BQA. Nonetheless, results obtained with ASC are contradictory since, under certain conditions, ASC was demonstrated to act as a pro-oxidant, quickly co-oxidating with HGA and leading to additional production of reactive oxygen species (ROS) [3]. We found that HGA and ASC significantly affect the levels of proteins with specific functions in protein folding, cell organization and, notably, stress response and cell defence. Either HGA or ASC induced oxidative stress and, specifically, carbonylation of proteins was demonstrated together with the production of high-molecular weight carbonylated protein aggregates.

Altogether, evidences produced in this paper support the hypothesis of HGA-induced oxidative stress mediated by the production of ROS and benzoquinone acetate after auto-oxidation of HGA. Our findings can lay the basis towards the settling up of more sophisticated models to study AKU and ochronosis.

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1.23

EVALUATION OF ANTI-OXIDANT TREATMENTS IN AN IN VITRO MODEL OF ALKAPTONURIC OCHRONOSIS

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Alkaptonuria (AKU) is a rare genetic disease associated with deficient homogentisate 1,2-dioxygenase (HGO) activity in the liver [1]. This leads to the accumulation of homogentisic acid (HGA) and its oxidized/polymerized products in connective tissues, which in turn become characterized by the presence of melanin-like pigments (ochronosis) [2]. Since at present further studies are necessary to support the use of drugs for the treatment of AKU and ochronosis, we investigated the effects of various anti-oxidants in counteracting melanin-like pigmentation and oxidative stress related to HGA and its metabolites.

We settled up an *in vitro* model using human serum treated with 0.33 mM HGA, which is in the range of circulating HGA in AKU patients' serum [3], and tested the anti-oxidants ascorbic acid, N-acetylcysteine, phytic acid, taurin, ferulic acid, and lipoic acid. Monitoring of intrinsic fluorescence of HGA-induced melanin-like pigments was used to evaluate the efficacy of compounds in preventing or delaying the ochronotic phenomenon. Carbonylation and thiol oxidation were investigated through SDS-PAGE and Western Blot to assess oxidative post-translational modifications of serum proteins.

Our model allowed to prove the efficacy especially of phytic acid, taurine, lipoic acid and ferulic acid in counteracting the production of HGA-induced melanin-like pigments and protein oxidation induced by HGA and its metabolites. Our results allow to open new anti-oxidant therapeutic strategies to treat alkaptonuric ochronosis.

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1.24

SERUM "ALBUMINOME" MALDI SIGNATURES FOR EARLY DETECTION AND STAGING OF HEPATOCARCINOMA: PRELIMINARY RESULTS

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Serum/plasma proteome contains hidden diagnostic information for a variety of diseases [1]. In the realm of biomarker discovery we have the possibility nowadays to simultaneously monitor multiple proteins or peptides in serum for diagnostic and prognostic purposes [2]. However, several drawbacks affect the stability of protein biomarkers in blood, resulting in high variability and poor reproducibility; it is therefore necessary to protect proteins from degradation and to set statistical procedures able to deal with the high variability of these analysis.

In the present study we developed a protocol for enrichment and MALDI-TOF analysis of low-abundance peptides bound to albumin, exploiting the documented protective feature of albumin on hypothetical biomarkers [3]. Profiling of albuminome peptides was performed using 40 µL serum samples from 3 classes of patients (cirrhosis, HCCs, advanced HCCs, 5 subjects per class). A quality assurance protocol was implemented and 3 samples for each subject were processed. Overall, 206 patient spectra and 236 quality control spectra were collected; a Random Forest machine-learning algorithm [4] was used to classify these datasets.

Although the mean inter-subject coefficient of variation was in the range 73.1%-110.0% among the three study groups, the Random Forest procedure correctly classified 440/442 spectra, with an out-of-bag error of 0.45%.

Our data suggest that, despite the large analytical variability, the Random Forest approach can properly differentiate spectra from different classes of patients. Based on these promising results, an enrolment of a greater number of patients is currently in progress to validate our approach in a large-scale study.

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1.25

PROTEIN STABILITY STUDIES: STANDARDIZATION OF PRE-ANALYTICAL PHASE FOR PROTEOMIC STUDIES IN HUMAN BLOOD SAMPLES (SPIDIA-PROT)

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Aim of the European project "SPIDIA-PROT" is the evaluation of protein stability in a blood sample to be processed for proteomic analysis. The study is designed to systematically test the effects of pre-analytical variables as processing time and type of collection tube that can potentially impact the outcome of results and to provide guidance on sample handling issues.
Whole blood from normal donors was collected into 3 different types of evacuated blood collection tubes (K_2-EDTA; Serum and Plasma plus Inhibitors). The specimens were processed at 3 time points (0h, 4h and 24h). Since in the 2D-DIGE we can load up to 3 labelled samples onto the same Immobiline DryStrip gel, the experimental variation is limited. The images of gels were analyzed by software that automatically locates and analyzes protein spots (threshold = 2-fold increase and decrease). In order to identify those differentially expressed proteins, we can prepare a Picking gel, so selected spots will be excised, in situ digested and subjected to MS analysis for protein identification. For the meantime, most significant changes are related to fibrinogen gamma chain between plasma and serum samples. Furthermore, in the tube containing Inhibitors it's possible to observe a greater recovery and preservation of proteins. Next step will be send aliquots of each sample to Rules Based Medicine that will analyse them and provide the most comprehensive menu of quantitative, multiplexed immunoassays (89 Antigens).

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1.26

ACETYLOME AND PHOSPHOPROTEOME MODIFICATIONS IN IMATINIB RESISTANT CHRONIC MYELOID LEUKAEMIA CELLS TREATED WITH VALPROIC ACID

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Chronic myelogenous leukaemia (CML) is a neoplasia associated with the specific chromosomal abnormality t(9;22) (q34;q11), which produces the tyrosine kinase BCR/ABL, a constitutively activated fusion oncoprotein BCR/ABL(1). CML therapy has been revolutionized by the introduction of imatinib, a tyrosine kinase inhibitor. In CML patients, imatinib resistance is due to point mutations of BCR/ABL kinase domain, amplification of the bcr/abl genomic locus but also because of bcr/abl independent mechanisms(2). We analysed whether enhancement of sensitivity to imatinib by valproic acid (VPA), an histone deacetylase (HDAC) inhibitor, is accompanied by significant proteome modifications in Bcr/abl positive human cell lines LAMA sensitive (S) and resistant (R) to imatinib. Total cell proteins were separated by 2D-electrophoresis; gels were immunoblotted with anti-pan-acetylated and anti-phosphotyrosine antibodies or stained by blu Comassie, followed by spot-matching between immunoblots and Comassie stained gels and analysed by MALDI-TOF mass spectrometry. In LAMA R cells global acetylation was increased by VPA, and, further addition of imatinib, both acetylation and number of acetylated spots decreased. Twenty-three acetylated proteins were identified by mass spectrometry. In LAMA R five of these proteins were significantly acetylated by VPA respect to untreated controls as HSP90 beta. VPA plus imatinib increased acetylation of TCP-1 and hnRNP L. When considering protein phosphorylation, imatinib plus VPA produced, mostly in resistant CML cells, a considerable decrease of that of HSPs and hnRNP A1 and A2. These data demonstrate that VPA is able to modify acetylome and phosphoproteome of CML cells and that in parallel it could restore sensitivity to imatinib.

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1.27

PROTEOMIC MODIFICATIONS INDUCED BY 5-AZACITIDINE AND DECITABINE IN AML1/ETO POSITIVE LEUKEMIA CELLS

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Acute myeloid leukemia (AML) is a heterogeneous disease which has been associated with recurrent gene rearrangements as t(8;21) (q22;q22) that leads to formation of AML1/ETO chimeric gene. This chimeric protein recruits a DNA HDAC/DNMT repressor complex that induced CpG islands hypermethylation by DNA Methyltransferase and histone deacetylation by HDAC. These epigenetic alterations regulated gene expression and are also frequently involved in changes in transcriptional activity of oncogenes (1).
We analysed the effects of two DNA methyltransferase inhibitors, 5-Azacitidine and Decitabine, on the proteome of AML1/ETO positive cells.
Total cell protein extracts of treated and untreated cells were separated by two dimensional electrophoresis. The 2D gels were stained by Blu Comassie and analysed by adequate software (Image Master TM Platinum). Untreated and treated gels were matched in order to choose interesting spots that were excised from the gels and identified by MALDI-TOF MS analysis (PMF and MS/MS).
We identified 39 proteins from cells treated with 5-Azacitidine: 3 proteins were induced, 4 proteins inhibited and 4 proteins reduced by 5-Azacitidine.
After treatment with Decitabine we identified 29 proteins: 1 protein was induced, 1 protein inhibited, 2 proteins overexpressed and 6 proteins reduced by Decitabine.
Moreover, we identified common proteins with different spot distribution, probably because of the presence of different isoforms or post translational modifications of the same protein.
Proteomic analysis is a new method that allows analyze changes in protein expression of acute myeloid leukaemia cells and may also help to identify potential biomarker as specific targets for therapies.

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PROTEOMIC MODIFICATIONS INDUCED BY VALPROIC ACID (VPA) AND ITF2357 IN AML1/ETO POSITIVE LEUKEMIA CELLS

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Acute myeloid leukemia (AML) is an heterogeneous disease that frequently presents molecular alterations affecting core binding factor (CBF) genes, as the translocation (8;21) (q22;q22) that leads to formation of AML1/ETO chimeric gene recruiting to regional histone deacetylase/DNA methyltransferase/co-repressor protein complex (1).
We have previously demonstrated that CBF-AMLs, because of their characteristic molecular alteration, are specifically sensitive to low dose HDAC inhibitors (2).
In this study, we analysed the effects of two HADACi, Valproic acid (VPA) and ITF2357 on the proteome of AML1-ETO-positive cells.
Total cell protein extracts of treated and untreated cells were separated by two dimensional electrophoresis. The 2D gels were stained by Blu Comassie and analysed by adequate software (Image Master TM Platinum). Untreated and treated gels were matched in order to choose interesting spots that were excised from the gels and identified by MALDI-TOF MS analysis (PMF and MS/MS).
We identified 13 proteins after treatment with VPA: 3 proteins were induced, 2 proteins inhibited, 4 proteins reduced and 2 proteins overexpressed by VPA.
After treatment with ITF2357 we identified 23 characteristic proteins spots including 1 protein found only in treated cells (Endoplasmin) and 22 more expressed in treated cells, as hnRNP F/H involved in apoptosis regulation (3) .
The effects of HDACi appeared significant in AML1-ETO-positive cells where the stability of DNMT/HDAC repressor complex binding to DNA was reduced by these drugs. Thus, the treatment with HDACi, possibly in combination with hypomethylating agents may specifically interfere with the molecular mechanisms of leukemogenesis.

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OCCURRENCE OF S100A7 IN A LARGE SAMPLE-SET OF BREAST CANCER TISSUES

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The S100 proteins are generally small (10-12 kDa) and acidic proteins, with cellular and occasional extracellular location. Altered expression levels of S100 proteins have been associated with a number of human diseases included cancer. Among the S100 proteins, great attention has been given in the last years to the S100A7, also named "psoriasin" because it was firstly identified as a highly up-regulated and partially secreted protein in psoriatic skin.
S100A7 protein expression has been associated with carcinogenesis of certain type of tumors such as skin, squamous cell carcinomas and large cell carcinomas of the lung, bladder, oral squamous cell carcinoma, as well as some breast tumors.
In the present study we aimed to detect the occurrence of S100A7 protein in the proteomic maps of 100 breast cancer tissues. Our results showed a sporadic presence and high expression level of S100A7 protein among patients, which suggests the possible role of this protein for patient stratification. S100A7 expression levels were also confirmed by western blot and immunohistochemistry analyses among the 100 selected patients. We also verified the absence of the S100A7 expression in the non-tumoral tissues adjacent to the primary tumor. Finally, the protein-interactome for the S100A7 was performed with the Ingenuity Pathways Analysis system. We believe that this information may substantially contribute to the progress of protein profiling of breast cancer and help to organize subclasses for clinical applications.

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1.30

PROTEOMIC ANALYSIS OF SERUM FROM PATIENTS WITH HIV- ASSOCIATED LEUKOENCEPHALOPATHIES FOR THE IDENTIFICATION OF POTENTIAL BIOMARKERS OF DISEASE

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Progressive Multifocal Leukoencephalopathy (PML) is a demyelinating disease of Central Nervous System (CNS) caused by JC Virus (JCV) in immunocompromised and HIV-positive patients. After the introduction of Highly Active Antiretroviral Therapy (HAART) for HIV treatment, a novel leukoencephalopathy, not associated to a known human virus, has been observed and termed Not Determined Leukoencephalopathy (NDLE),
Since the etiopathogenesis of NDLE is unclear and the discrimination between PML and NDLE is currently based on the detection of JCV DNA within CSF, we performed a proteomic study on PML and NDLE sera for identification of diagnostic and prognostic markers of disease by means of Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF).
Sera samples from five PML and five NDLE patients were collected. The sera were depleted of albumin and IgG and the resulting serum proteins were separated on two dimensional gel electrophoresis (2DE). Data were analysed using Ludesi Redfin Software and the differentially expressed spots were cut from gels and tryptic digested. Peptides were purified with C18 Zip-Tip and spectra were obtained with a Microflex-LRF MALDI-TOF. The mass lists obtained from chromatograms were used for database search on Mascot (Matrix Science) and Aldente (Expasy).
Four proteins have shown a different expression between the two case groups. We have detected an increase of hemopexin, haptoglobin-beta chain, wnt2B in PML sera and an increase of vitamin D-binding protein (DBP) in NDLE sera.
These preliminary data, that will be validated by increasing the number of studied samples and using western blotting, are at this moment very promising.

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1.31

PROTEOMIC ANALYSIS OF CELLULAR RESPONSE TO NOVEL PROAPOPTOTIC AGENTS RELATED TO ATYPICAL RETINOIDS IN HUMAN OVARIAN CARCINOMA CELLS IGROV-1

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Novel agents characterized by the scaffold of the atypical retinoid ST1926, but containing different chemical functions (carboxylic or hydroxamic acid) exhibit potent proapoptotic activity. In the present study, we show that the treatment of ovarian cancer cell line IGROV-1 with three selected compounds of the novel series determines a strong inhibition of proliferation mainly due to apoptotic cell death. In an effort to understand the mechanism of action of these compounds, we performed a proteomics analysis of IGROV-1 total lysates and nuclear extracts. Using this approach, we found that deregulation of calcium homeostasis, oxidative stress, cytoskeleton reorganization, and deregulation of proteasome function may represent important pathways involved in IGROV-1 cells. The most prominent effect was downregulation of factors involved in protein degradation. In addition, we identified proteins specifically modulated by each treatment, including prohibitin and co-chaperone P23, pre-mRNA splicing factor SF2p32 and clathrin light chain, as well as Far upstream element (FUSE) binding protein 1 and DNA-binding protein B. This study by identifying proteins modulated by novel atypical retinoids provides insight into critical aspects of their mechanism of action.

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PROTEOMIC ANALYSIS OF SYNOVIAL FLUID FROM EQUINE OSTEOCHONDROTIC JOINTS.

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Osteochondrosis (OC) is an important joint disorder characterised by a focal disturbance of enchondral ossification that occurs in humans, horses and other animal species. Even though several factors associated with the changes in cartilaginous structures, such as heredity, rapid growth, anatomic conformation, trauma, and dietary imbalances, have been considered as causes of OC, its etiology and pathophysiology remain not yet completely elucidated. Consequently, diagnosis in the early stages of OC is very difficult.

In order to understand the molecular factors involved in the pathogenesis of OC and to identify expressed proteins that might become a valuable tools to be used as diagnosis biomarkers, we analysed the synovial fluid protein pattern in joints with OC and controls. Thus, synovial fluid samples were collected from healthy, OC hock and fetlock joints of horses, after clinical evaluation. Proteins were separated by two-dimensional gel electrophoresis; spots were detected by colloidal Comassie blue staining; image analysis and gel comparison were carried out by PD QUEST software. Differentially expressed proteins were alkylated, digested with trypsin and identified by using MALDI-TOF peptide fingerprinting and nLC-ESI-LIT-MS/MS experiments. They corresponded to proteins involved in bone development, stabilization and protection of extracellular matrix, as well as in supplying nutrients.

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1.33

TISSUE PROTEIN PROFILE OF CLEAR CELL RCC BY 2D-DIGE AND MS: A PROTEOMIC OVERVIEW OF FUNCTIONAL AND NETWORK SIGNATURES

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Renal cell carcinoma (RCC) is the most common neoplasm in the adult kidney and one of the most therapy-resistant carcinomas. Patients' outcomes with surgery are primarily dependent on tumour characteristics. This suggests that a better understanding of changes in protein expression levels and their functional pathways is a significant clinical issue in order to provide insight to its pathogenesis and its therapy resistance mechanism. Therefore we have undertaken a comparative proteomic analysis carried out with 2D-DIGE technique combined with mass spectrometry between 20 clear cell RCC lesions (ccRCC), versus 20 autologous normal kidney epithelium (renal cortex). Subsequently a computational analysis of pathways and cell signalling networks related to the selectively up- or down-regulated proteins, using Ingenuity Pathway Analysis and other functional annotation tools as Panther, DAVID and WebGestalt, was provided in order to identify possible biological processes and molecular functions altered in ccRCC and the focus molecules associated. Molecular characterization using 2D-DIGE and mass spectrometry let us to detect a group of proteins which are dysregulated in ccRCC (39 different human protein isoforms more expressed in tumour and 124 in normal counterparty). Bioinformatic analysis performed on these proteins have confirmed the complex molecular nature of this malignant tissue which involves different metabolic pathways which will be discussed in the present work. Therefore this approach could be a first step towards elucidation of the metabolic signatures of ccRCC and key biological processes altered or disrupted in this tumour and a challenge to integrate data and tools.

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1.34

PROTEOMICS INVESTIGATION OF PLATELETS AFTER INHIBITION AND CARDIOVASCULAR EVENTS (APICE PROJECT)

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Dual antiplatelet therapy (aspirin and clopidogrel), aimed to inhibit platelet reactivity, is the recommended standard of care for reducing the occurrence of major adverse cardiovascular events (MACE) in patients with acute coronary syndromes (ACS) undergoing percutaneous coronary intervention (PCI) with stent implantation. A large body of evidence exists demonstrating that nonresponsiveness to clopidogrel (ADP-RPR) and/or aspirin (AA-RPR) is associated with increased risk of adverse clinical events [1,2]. In the preliminary phase of the "Activity of Platelets after Inhibition and Cardiovascular Events" (APICE) project, we have started the proteomic analysis of platelet protein profiles from ACS patients undergoing PCI with stent implantation on dual antiplatelet treatment. Total protein extracts obtained from platelets of patients with or without residual platelet reactivity (RPR) were analyzed at the time of the acute event. We have identified more than 1000 plasma proteins and some differentially modulated proteins among patients with or without RPR. Interestingly, significant differences were found either between patient with or without RPR and between patients with AA-RPR or ADP-RPR. Many of the differentially expressed proteins are directly involved in adherence and activation of platelet aggregation (such as platelet/endothelial cell adhesion molecule, von Willebrand factor). Other proteins are involved in the platelet cytoskeleton organization (such as filamin A, tubulin beta 1). In conclusion, our preliminary proteomic profiling of platelets identifies extensively the total proteome content and differences among patients with or without RPR by ADP or AA underling different actors that might play a role in the pathological mechanisms.

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1.35

PROTEOMIC ANALYSIS OF BRUGADA SYNDROME

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Brugada syndrome (BS) is an polygenic inherited cardiac disease characterized by life threatening arrhythmias and high incidence of sudden death. In the family enrolled in the present study, the disorder is caused by Q1118X-mutation in the SCN5A gene, encoding the cardiac sodium channel. 2D-PAGE was used to investigate specific changes in the plasma proteome of BS affected patients and family members sharing the same gene mutation, compared to healthy controls, with the aim to identify potentially specific disease biomarkers.

In order to reduce plasma sample complexity, we used the combinatorial hexapeptide ligand libraries (ProteoMiner TM, Biorad) [1]. The use of the beads prior 2D-PAGE enabled detection of many new protein spots and increased resolution and intensity of low abundance proteins.

Approximately 900 protein spots were detected in each gel. Proteins, whose expression was significantly different among the two groups, were excised, trypsin-digested and analyzed by LC-MS/MS.

Our data showed that the levels of several proteins were significantly altered in BS patients compared with controls. In particular, Apolipoprotein E, Prothrombin, Vitronectin, Complement-factor H, Vitamin-D-binding protein, Voltage-dependent anion-selective channel protein 3 and Clusterin were considerably increased in plasma sample of BS patients, whereas Alpha-1-antitrypsin, Fibrinogen and Angiotensinogen were considerably decreased; moreover, post-translational modification of Antithrombin-III was detected in all affected individuals.

On the light of these results, we hypothesize that these proteins might be considered as potential markers for the identification of disease status in BS. Further analysis is undergoing in our laboratory in order to validate these findings in a larger number of cases and to elucidate the pathogenetic role of these proteins in this specific cardiac disease.

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PROTEOMIC PROFILING OF MULTIPLE MYELOMA – THE EFFECT OF BORTEZOMIB ON MYELOMA CELL LINE

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Introduction: Multiple myeloma (MM) is still an incurable disease characterized by the clonal expansion of malignant plasma cells. New anticancer drugs further improve prognosis
of myeloma patients. The aim of this study was to evaluate changes in protein expression
of myeloma cell line ARH 77 after bortezomib treatment.

Materials and methods: Myeloma cell line ARH 77 was treated with bortezomib (10 – 40nM) for various periods of time (24 and 48 hours). The proteins contained in total myeloma cell lysate were separated by 2-dimensional polyacrylamide gel electrophoresis (2-DE). The gel patterns were visualized by tetrathionate-silver nitrate staining for analytical purposes or
by SYPRO Ruby. The differentially expressed proteins between the untreated and treated cell lines were excised and identified by mass spectrometry.

Results: There were analyzed 30 proteins differentially expressed between treated and control cells; total of 14 protein spots were upregulated: proteins involved in regulation of apoptosis, chaperons/stress related proteins, proteolysis of ubiquitin/protein degradation and cytoskeleton proteins. Sixteen protein spots were downregulated: proteins involved
in synthesis, regulation of apoptosis, chaperons/stress related proteins, regulation of cell cycle proteins, proteins connected to glycolysis and proteolysis of ubiquitin/protein degradation and antioxidant/redox proteins.

Conclusion: We identified 30 proteins altered in myeloma cells after various exposure of to bortezomib. This proteomic approach can contribute to elucidation of mechanisms of new anticancer drugs action.

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1.37

SHOTGUN PROTEOMICS ANALYSIS AND FUNCTIONAL PATHWAY MINING: POWERFUL TOOLS FOR UNDERSTANDING DRUG RESPONSE IN CANCER

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The synthetic compound *6*-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX) is a promising anticancer drug that inhibits GSTP1-1 activity. Its binding triggers the dissociation of the GSTP1-1-JNK complex and leads to the activation of the JNK pathway thus inducing apoptosis. The compound is cytotoxic toward the P-gp and MRP1 over-expressing cells resistant to the most common anti-neoplastic agents[1]. Moreover NBDHEX demonstrated a high level of activity on both sensitive and resistant osteosarcoma cell lines[2]. We performed a comparative proteomic analysis by label-free LC-MS^E of U-2OS human osteosarcoma cell line before and after 24 hours of treatment with 5µM NBDHEX to provide insights into cell cycle arrest and apoptosis mechanisms caused by drug exposure. Relative quantitation analysis revealed a total of 33 significant differentially expressed proteins between the two conditions. Bioinformatics tools were combined to the shotgun experiment in order to obtain an extensive system biology overview of different cellular networks involved in drug response. The unsupervised in silico analysis revealed the possible involvement of TRAF family proteins. In particular we focused our interest on a member of this family, TRAF2, which is reported to be physically associated with GSTP1-1 in human cervical carcinoma HeLa cells. TRAF2 is an adaptor protein which mediates the signal transduction of different receptors and is required for the activation of the apoptosis signal-regulating kinase (ASK1), which in turn activates both MKK4/7–JNK and MKK3/4/6–p38 signalling pathways. The evaluation of the role of TRAF2 in the apoptosis signaling caused by NBDHEX is under investigation.

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1.38

SELDI-TOF/MS PROFILE OF FINE NEEDLE ASPIRATION FLUID OF THYROID : IS IT A POTENTIAL TOOL TO DISCRIMINATE FOLLICULAR PROLIFERATIONS?

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The identification and differentiation of rarely occurring thyroid cancer from frequently benign nodular thyroid disease is challenging. Although fine needle aspiration cytology (FNAC) represents the most sensitive and specific tool for the differential diagnosis of thyroid malignancy, there are important limitations. While 75% of FNAC reveal a benign, and 5% a malignant lesion, up to 20% of FNAC reveal follicular neoplasia for which surgery is the only method to differentiate between follicular adenoma, follicular carcinoma, and the follicular variant of papillary carcinoma. For this reason in the clinical setting, the introduction of markers would be helpful to discriminate the nature of different follicular proliferations. In the present study we perform a proteomic analysis of FNA in non-functioning thyroid nodules with a FNAC of follicular proliferation (n=20), carcinoma (n=20) and benign (n=40) by using SELDI-TOF/MS approach. The protein profiles of FNAs obtained from all samples by four different chips (CM10, Q10, IMAC 30 and H50), were analysed and the data of clusters of interest were processed by multivariate analysis (SIMCA P, Umetrics software) to reduce data dimensionality and segregate the samples based on their variability. Among the comparison of different classes 23 clusters (up and down regulated) were found with significant p values. The preliminary results suggest the potentiality of this analysis to discriminate malignant and benign samples in the cytological examination, as subsequently confirmed by histological analysis.

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PROTEOMIC ANALYSIS OF THE ANTI-ANGIOGENIC EFFECT OF LENALIDOMIDE ON PRIMARY BONE-MARROW ENDOTHELIAL CELLS ISOLATED FROM MULTIPLE MYELOMA-PATIENTS

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Multiple Myeloma progression depends on the interaction between malignant plasma cells and their permissive bone-marrow [BM]-microenvironment [1]. Immunomodulatory drugs, such as *Thalidomide* and *Lenalidomide*, have been shown to block several pathways important for MM progression, and represent effective anti-tumor/vessel agents [2]. Our study is focused on the identification of proteins potentially involved in the anti-angiogenic response elicited by *Lenalidomide* in bone-marrow endothelial cells (ECs) isolated from patients with active MM (MMECs). We performed a proteomic analysis (2-DE and MS/MS) of MMECs isolated through a lectin-based method from BM-biopsies of 2 patients with active disease and cultured with or without *Lenalidomide* 1.75μM for 72h. Consistent with the inhibitory effects observed upon exposure of MMECs to *Lenalidomide in vitro*, most of the identified proteins are angiogenesis-related agents controlling EC adhesion and invasiveness (LBP/p40), cytoskeletal dynamic rearrangements (Vimentin, Keratin2C1, Septin-2, γ-Actin) as well as coordinated events at the interface between cytoskeleton and membrane biology (cell-shape, cell division, polimerization of F-actin microfilaments, transport of organelle or ribosomal subunits during protein biogenesis). Alteration of specific enzymatic activities (DLDH, DHE3, PNPH and ENOA) appears indicative of mitochondrial dysfunction and an oxidative stress response associated to reactive oxygen species (ROS) production and apoptotic effects of *Lenalidomide* in MMECs. Up-regulated levels observed for the proteasome subunit beta4 (PSB4) after the treatment with *Lenalidomide*, could be part of a compensatory response to increase changes in energy metabolism**,** protein's clearance and turnover, hence cell damage.

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TOP DOWN PROTEOMIC PROFILING OF CEREBROSPINAL FLUID BY LC-MS IN ASSOCIATION WITH PEDIATRIC BRAIN TUMORS

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The proteomic analysis of CSF offers a unique window for studying pathologies and disorders of the SNC and was demonstrated of great biomedical relevance for the identification of disease biomarkers [1-6]. Only very few papers report proteomic studies of CSF in association to brain tumors and no one is in relation to pediatric brain tumors.
This work presents the preliminary results obtained by LC-MS proteomic analysis of CSF samples of pediatric patients affected by posterior cranial fossa brain tumors.
CSF samples pre- and post- surgical removal of the tumor were analysed by liquid chromatography in coupling with tandem mass spectrometry after a simple pretreatment procedure to remove the most abundant proteins. The proteomic analysis was performed using a top-down approach in which proteins are analysed in their naturally occurring form and directly fragmented during MS (MS^2 or MS^n) analysis. The obtained LC-MS profiles were compared for the identification of potential biomarkers of disease.
Preliminary results showed interesting differences in the LC-MS profiles of specific small peptides in CSF that exhibited a significative behaviour in the different phenotypes, i.e. in the presence and absence of the tumor and in the controls. These bioactive peptides were identified as fragments specifically produced in SNC from a parent protein. These results, if confirmed in a wider number of samples, could lead to the identification of peptides biomarkers of revelant clinical application both in the monitoring the success of the surgical therapy and the evaluation of disease prognosis.

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1.41

A PROTEOMIC ANALYSIS OF RED BLOOD CELL CYTOSOL PROTEINS IN DOWN SYNDROME PATIENTS

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Down's syndrome (DS) is the most frequent chromosomal alteration in man. It has been hypothesized that an increase in oxidative stress in patients with DS would account for the appearance of different diseases, such as accelerated cell aging, cellular mutagenicity, and the neurologic disorders that often occur in these patients. Red Blood Cells (RBCs) are considered important regulators of oxidant reactions in their surroundings. They not only are excellently equipped to handle intracellular oxidative stress, but also account for much of the antioxidant capacity of the blood. In light of these considerations, the present study was designed to evaluate the expression of RBCs cytosol protein levels of patients with DS (n = 21, 16 women and 5 men, age range18-35 yrs) compared to apparently healthy controls (n = 18, 14 women and 4 men, age range 18-35 yrs). RBCs cytosols were analyzed using the ProteoMiner Protein Enrichment Kit to detect low-abundance proteins which were then quantified by the ICPL (Isotope Coded Protein Labelling) technique. The results revealed a significant change in the expression level of several proteins associated to cytoskeletal, stress response and metabolic function. Among these, heat shock protein (HSP) 90 and obg-like ATPase 1 (OLA1) appeared to be deregulated in DS. Although preliminary, the data obtained are suggestive for a potential role of proteomic analysis of RBCs cytosol in the evaluation of stress response in patients with DS.

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1.42

A PROTEOMIC APPROACH TO SEARCH POTENTIAL BIOMARKERS USEFUL IN COLON CANCER DIAGNOSIS

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Colon cancer is a preventable, treatable cancer when detected at the premalignant or early stages. Even if screening is necessary to detect precancerous and early stage colorectal cancer it is still not uniformly accepted by the eligible public.
The purpose of this study is to apply a proteomic approach to search potential proteins sufficiently sensible and specific to serve as a useful screening biomarkers for early detection of colorectal carcinomas. Our idea is to search proteins differentially expressed in the washing fluid of colorectal tract after surgical resection, to obtain a mixture of proteins deriving from secretion of tumoral epithelial cells and then potentially involved in the pathological progression of tissue.
Samples of washing fluids were obtained at surgery from 32 patients submitted to colon resection for adenocarcinoma or carcinoma while the respective controls were obtained from washing of healthy section. Samples were immediately centrifuged, concentrated and resulting protein pellets were obtained after TCA precipitation. After 2D separation the protein patterns of colon cancer and normal samples were compared and a total of 20 protein spots were found to be differentially expressed, each exhibiting $\geq$ 2 fold-change (either increase or decrease) of mean value spot intensity in the cancer with respect to normal samples. At this time the proteins of interested have been submitted at mass spectrometry to obtain protein identification. Our preliminary results suggest the applicability of two dimensional electrophoresis at our samples and suggest that washing fluids could be a starting point to study the presence of potential markers implicated in tumor onset and progression.

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1.43

DIFFERENT ISOFORMS OF S100A9 (CALGRANULIN B) DETECTED IN HUMAN PRE-TERM NEWBORN SALIVA

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S100A9, also named calgranulin B, belongs to the family of S100 calcium-binding proteins. S100A9, together with S100A8, is abundant in neutrophils and exerts both extracellular and intracellular functions. It forms hetero-complexes with S100A8 and its activity differs between free and complex form. Among various functions, the dimer S100A8/S100A9 is involved in wound repair by the reorganization of keratin cytoscheleton [Heizmann, C.W., Fritz, G., Schafer, B.W. Frontiers in Bioscience 2002, d1356-1368]. A HPLC-ESI-MS proteomic study carried out by top-down approach evidenced in human pre-term newborn saliva the presence of four different isoforms of calgranulin B: a long form (S100A9: 13153 Da), a short form (S100A9*: 12689 Da) and their phosphorylated derivatives (S100A9 phos: 13233 Da; S100A9* phos: 12769 Da). High resolution MS-MS experiments allowed establishing that, with respect to the sequence reported in Swiss-Prot data bank (P06702), the long form was acetylated on the N-terminus after a methionine loss, while the short form was acetylated on the N-terminus after the loss of the first five amino acid residues (MTCKM). Phosphorylation involves the penultimate residue of the sequence (threonine) of both the isoforms. The amount of the four isoforms was established by eXtracted Ion Current (XIC) MS procedures. The amount decreased as a function of post conception age (PCA) (N = 61), but with different trends. S100A9 and its phosph. derivative were the predominant isoforms between 195 and 230 days of PCA. Because their concentration decreased more rapidly than that of S100A9* and its phosph. derivative, after 230 days of PCA the latter become the predominant isoforms. S100A9 and its phosph. derivative were not detectable in whole saliva of at-term newborns and of adults, while S100A9* and its phosph. derivative were detectable in these samples, even though at a concentration about two order of magnitude lower than that measured in pre-term newborns of 195-210 days of PCA. The percentage of the phosphorylated derivatives was 25 (±7) % for both isoforms and it did not change as a function of PCA, suggesting that the kinase responsible for this PTM is fully active in the foetus before 195 days of PCA. Some speculations on the role of the isoforms during foetal development will be presented.

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COMPREHENSIVE PROTEOMIC OVERVIEW OF EPS-URINE FOR THE IDENTIFICATION OF NOVEL PROSTATE CANCER BIOMARKERS

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Prostate cancer (PCa) is the most prevalent cancer in the Western male population. Prostate-specific antigen (PSA) is the most widely-used marker for PCa detection, although it is often unable to distinguish between PCa and nonmalignant prostatic diseases (i.g. BPH). Thus, novel PCa biomarkers for improved early detection are urgently needed. Since proximal fluids are conceptual discovery sources for cancer biomarkers, we used expressed prostate secretions, a prostate proximal fluid collected following a digital rectal prostate massage in voided urine post-exam (termed: EPS-urine). Eleven EPS-urine samples (five low-grade PCa and six BPH) were analyzed by extensive MudPIT-based proteomics. A rigorous protein identification criterion was employed and more than 1000 unique proteins were identified. This extensive compendium was compared to other available datasets. To obtain a systematic overview of the functional categories of proteins expressed in EPS-urine, we performed GO enrichment analyses and determined that proteins involved in immune response and catabolic processes, proteins with binding properties and proteins with vesicle, extracellular and membrane localization were significantly over-represented in the EPS-urine proteome in comparison to the UniProt database. We also found that among the identified proteins more than 50% contained a predicted signal peptide and about 43% at least one predicted transmembrane domain. Finally, we compared the proteome expression profile of EPS-urine samples from PCa and BPH patients by spectral counting. We selected a group of 59 proteins with significant differences ($p<0.05$) between PCa and BPH samples. Moreover, to verify the specificity of these putative biomarkers, we compared the EPS-urine proteome to five PCa urine, five kidney cancer urine and five bladder cancer urines. The validation of these data by ELISA and Western Blot assays allow us to propose a panel of novel biomarkers for PCa. Since EPSurine is a prostatic biofluid easily collectible during the screening procedures preceding any aggressive treatment of PCa, such as prostatectomy, we believe that these data may have a high impact on clinical practice.

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PROTEOMIC AND FUNCTIONAL CHARACTERIZATION OF IMATINIB RESISTANT CHRONIC MYELOGENOUS LEUKEMIA CELLS

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The oral administration of imatinb mesylate (IM), a specific reversible inhibitor of BCR-ABL kinase, has dramatically changed the therapeutic approach towards individuals diagnosed with chronic myelogenous leukemia (CML), inducing a significant hematological and cytogenetic remission in high percentage of patients in chronic-phase CML. However, when disease proceeds to blast crisis most of patients develop resistance to this drug. The aim of our study was the understanding of biologic consequences of BCR-ABL over-expression, one of most frequent cause of IM-resistance. Thus we performed a proteomic study using as *in vitro* model the LAMA84R cell line in which the IM-resistant phenotype is due to Bcr-Abl gene amplification and to the consequent increase of oncoprotein levels. Therefore, we employed two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) in order to define the protein profile associated to resistant phenotype. The comparative proteomic analysis between LAMA84R and the IM-sensitive counterpart (LAMA84 cells) showed that the IM-resistance acquisition was correlated with changing in expression levels of 33 protein spots (26 were over-expressed in LAMA84R cell and 7 in LAMA84 cells). The GO terms analysis showed that about 31% of the proteins over-expressed in the resistant cells belonged to the category of proteins involved in anti-apoptosis pathway. Moreover, analysis of 33 differential proteins in the context of a *Protein-Protein Interaction* network (by using the freely available web-based tool PPI *spider*) revealed that 29 of them are mapped to a significant inferred protein network ($p < 0.01$), in which HSP70 had a central position, interconnecting different and apparently unrelated biological processes. Finally, it was interesting to observe that some proteins associated to the resistant phenotype (such as HSP70, HSP27 and Annexin 1) were inhibited by Carboxyamidotriazole (CAI), a drug studied in our laboratory to treat IM-resistant CML. These data indicated that CAI may determinate the reversion of resistant phenotype, as supported by functional assays.

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INTRAUTERINE GROWTH RESTRICTION: A PROTEOMIC STUDY

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Intrauterine Growth Restriction (IUGR) is the failure of a fetus to reach its genetically predetermined growth potential, and can be due to maternal, fetal, placental or external factors. IUGR is associated not only with increased perinatal mortality and morbidity, but also with enhanced morbidity and metabolic abnormalities later in life. In this study we have compared protein profiles of umbilical cord serum from IUGR and appropriate for gestational age full term (AGA) newborns. In order to detect low abundant proteins, samples (10 for each experimental group, IUGR and AGA) have been albumin-depleted and then analysed by 2D-PAGE coupled to RP-HPLC-MS/MS. We found modulated proteins associated with IUGR that may be informative of the pathological mechanism. In addition the differentially expressed proteins might reveal potential markers able to prevent postnatal complications, or useful from the legal medicine point of view. In parallel, an untargeted metabolomic approach carried out in cordonal sera from the two groups has proved to significantly discriminate the two groups.

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ANALYSIS OF EXHALED BREATH CONDENSATE: IS SELDI-TOF MS AN APPROPRIATE PLATFORM TO DISCRIMINATE COPD FROM HEALTHY SUBJECTS ?

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The search for biomarkers that may aid diagnosis or define specific disease subgroups of Chronic Obstructive Pulmonary Disease (COPD), an heterogeneous disorder characterized by progressive air flow limitation, has emerged as a crucial way to understand the molecular mechanisms of this severe pathology. Interest in non-invasive tests to study these biomarkers led to consider exhaled breath condensate (EBC) an attractive candidate for monitoring alterations in the respiratory tract. Aim of this work was to reveal in EBC patterns of proteins that could eventually distinguish patients from controls. SELDI-TOF MS was thus applied to EBCs collected from 70 subjects grouped into four distinct groups: non-smokers (NS), healthy smokers (HS), patients with pulmonary emphysema associated to α_1- antitrypsin deficiency (AATD) or affected by COPD with normal plasma AAT levels (COPD). Analysis with CM 10 chips resulted in protein profiles for all specimens investigated. Statistical analysis of patterns showed that the NS and HS groups were rather "homogeneous", the differences between them, in terms of peak number, being not significant ($p > 0.05$). Likely, no significant differences were noted between COPD and AATD patients. By contrast, an interesting significancy ($p < 0.01$) was found between NS/HS, considered as the "control group", and the group containing AATD/COPD patients. LC-MS/MS allowed to identify the SELDI *m/z* signals as structural, transfer, signalling/regulation, enzymes and inflammatory proteins. Quantification of these proteins in different groups will likely provide decisive progress in the identification of possible biomarkers of the disease.

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INVESTIGATION ON NATIVE PROTEOME OF RBCS CYTOSOL BY 2D CN SDS-PAGE

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Human erythrocytes' cytosol is one of the most studied proteome and in the last decade many works have been focused on the identification of unknown protein of this cellular fraction [1-3]. Nowadays, 1578 are the whole known proteins thanks to the combination of hexapeptide library beads and LTQ-Orbitrap mass spectrometer technologies [4]. However, an insight into their actual interaction and/or association to achieve all cellular physiological tasks is still needed. For this purpose, we have looked at cytsolic protein complexes and supercomplexes of RBCs cytosol by Clear Native polyacrylamide gel electrophoresis [5]. CN PAGE, a derivative technique of Blue Native PAGE [6], relies on a first dimension separation of proteins in native condition on a non denaturing gel. In a control cytosol (collected immediately after blood sampling) we have discovered at least 10 protein complexes with a molecular weight ranging from 50 to 700 kDa. Among them, the heaviest is a complex of haemoglobin and carbonic anidrase at around 670 kDa, followed by an homocomplex of haemoglobin and another carbonic anidrase/haemoglobin complex at around 500 and 440 kDa, respectively. The latter seems to be influenced somehow by blood aging, since its amount increases during the storage time. Moreover, coupling the non denaturing first dimension with SDS-PAGE has allowed to disclose the single members belonging to a certain protein complex.

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RETINOBLASTOMA: AQUEOUS HUMOUR ONCOPROTEOMIC

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Retinoblastoma (RTB) is the most common primary intraocular tumour in children, with an incidence of 1:15.000 births [1]. Although current therapeutic strategies have led to dramatic improvement of individual prognosis, retinoblastoma is still life-threatening when leaved untreated or in cases of late diagnosis, a condition of concern in developing countries [2]. This study is a preliminary approach to compare the basic protein composition of aqueous humour (AH) from retinoblastoma (RTB) patients with that from patients affected by cataract, used as controls (CTR). The study was carried out on 10 RTB patients (VB stadium according to REESE-ELLWORTH classification) who had undergone to ocular enucleation and 10 cataract patients after cataract surgery. From RTB patients, 5 presented secondary glaucoma associated (SG), and 5 without secondary glaucoma (NSG). Total protein concentration and electrophoresis pattern (2DE) were analysed in AH.

AH, in RTB patients, had significantly higher total protein concentration than controls ($p<0,01$); SG associated patients presented the highest total proteins concentration ($p<0,01$). Concerning to 2DE, the pattern of RTB patients was very different from CTR.

The present study indicates relationship between RTB and AH proteins and represent the first preliminary step for next investigations (including more accurate 2DE and mass spectrometry analysis). Other goal for the future is to clarify the role of specific proteins in tumour development and progression.

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SIZE HETEROGENEITY OF PLASMA APOLIPOPROTEIN-A1 IN NEPHROTIC SYNDROME

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ApolipoproteinA1 (apoA1) is a component of the high density lipoproteins (HDL) that regulates the transport of cholesterol between the liver and peripheral cells and modulates the removal of any excess of cholesterol from membranes. Any variation in apoA1 composition may modify the plasma lipid profile and be involved in atherogenesis.

We investigated apoA1 composition in plasma of 7 children with nephrotic syndrome utilizing non denaturing two-dimensional electrophoresis (Nat/SDS-PAGE). Mass spectrometry, western blot and pull down experiments were done to characterize proteins and define putative interactions. ApoA1 was resolved in 12 variants, 6 of which had a slightly lower molecular weight (17 KDa) and migrated on the same axes of the β of chain haptoglobin (Hpt). LC/Ms showed lack of any MS signals for their internal sequence region. Low molecular weight apoA1 were observed in carriers of different Hpt haplotypes (including one homozygous for the rare $\beta\beta\alpha1\alpha1$) ruling out any contaminant effect of co-migration of apoA1 with Hpt $\alpha2$ chain. Their presence in normal plasma was limited to subtle levels.

These findings show that apoA1 plasma profile in nephrotic syndrome is heterogeneous in terms of molecular weight. Low molecular variants lack internal structural domains and probably form macro-aggregates with hatoglobin. Fragmentation and transport of apoA1 may be involved in the general disorder of lipid metabolism that characterizes nephrotic syndrome.

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A PROTEOMIC APPROACH TO STUDY PARATHYROID GLANDS

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Diagnosis of parathyroid tumors may be difficult using histological features. By two-dimensional electrophoresis (2D) and mass spectrometry (MS) approach we performed a comparative proteome analysis to examine the global changes of parathyroid adenoma tissues protein profile compared to the normal tissues.

Thirty-two proteins were found to be differentially expressed, of which 24 resulted over-expressed. Proteins identified by 2D/MS/MS proteomics were classified into functional categories and a major change in terms of expression was found in proteins involved in response to biotic stimulus, cell organizations and signal transduction. The different expression of six proteins (peroxiredoxin-1 (PRDX-1), B box and SPRY domain-containing protein (BSPRY), mitogen-activated protein kinase 1 (MAPK-1), T-complex protein 1 subunit epsilon (CCT-5), 14-3-3 ζ/δ and parvalbumin) between adenoma and normal parathyroid samples was validated by immunoblot analysis. Moreover, after Ingenuity software application an assignment of biological processes and subsequent construction of networks was done. 14-3-3 ζ/δ is a key protein in the pathway of parathyroid adenoma and is linked to other proteins such as annexin A2, BSPRY, p53 and epidermal growth factor receptor (EGFR). In summary proteomic approach was able to differentiate protein profiles of normal parathyroid and parathyroid adenoma, to visualize global alterations of complex signal pathways and, finally, to identify a panel of several rather than single proteins differentially expressed, which might be early markers of parathyroid adenoma

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PAPILLARY THYROID CANCER: A NEW PROTEOMIC APPROACH FOR BIOMARKER DISCOVERY

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We evaluated the potential of a new approach for biomarker discovery in thyroid cancers. Wash-outs from fine needle aspiration were analyzed directly by linear MALDI-TOF-MS. Papillary carcinoma samples (6) were compared to benign thyroid lesions (24). We analyzed signals within the 5-20 kDA range. Peaks present in at least 50% of every group were subjected to Kruskall-Wallis analysis. Differential biomarkers were identified by LC- Q-TOF MS. Eleven peaks were different in the two groups and three of them had strong statistical power of discrimination. One Peak was characteristic of malignant lesions while the other two were found in benign lesions and were identified as Calgranulin B and Histone H2b respectively.

Since it is unlikely that there is a massive loss of H2b in tumours we investigated the possibility that the observed loss was due to post-translational modifications, particularly sumoylation and ubiquitination, that are compatible with the m/z shift. We therefore investigated the presence of these modifications comparing a cell line derived from a primary nodular goiter with cancer cell lines from different tumors: papillary carcinoma (BC-PAP), anaplastic carcinoma (SW1736) and follicular carcinoma (WRO). WB with anti-SUMO1 anti-body after immunoprecipitation with anti-H2b antibody confirms that differently from goiter cells Histone H2b is at least in part sumoylated in the papillary thyroid cancer cells line. In conclusion our data while still in part preliminary, show that our approach is capable of identifying potential cancer biomarkers. Moreover we found that sumoylation of histone H2B is associated with a tumour subtype and could play a role in tumour progression by altering gene expression.

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IDENTIFICATION OF NOVEL *cftr* EXPRESSION REGULATORY ELEMENTS

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Cystic fibrosis (CF) is the most frequent lethal genetic disorder among Caucasians. The disease is caused by *cftr* gene mutations on both alleles which induce alterations of the CF transmembrane conductance regulator (CFTR) protein, a cAMP-activated chloride channel located in the apical membrane of most secretory cells. In 92% of patients the mutations occurred within coding region of both alleles, but in 8% of CF the mutation of alleles is not identified. In these cases it is supposed that mutations might occur into highly conserved regulatory regions located into introns and/or in promoter sequences.

A specific region belonging either to the promoter or to the first intron of *cftr* and carrying point mutation(s) in a wide set of CF patients were cloned and used as "baits" in affinity chromatography experiments. The same experiment was carried out using non mutate oligonucleotide sequences as negative controls.

All the oligonucleotide baits were synthesized with a biotin tag, immobilised on Streptavidin derivatised magnetic beads and incubated with nuclear S9 cell extract in order to isolate the interacting proteins. The proteins fished out by the baits were separated by SDS-PAGE and identified by mass spectrometry methodologies and protein databases search.

A large number of interactors involved in the splicing mechanisms were identified in the experiments bound to both promoter and intron regions. Moreover, proteins differing between the interactors of mutated and wild type introns were also detected.

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A TOP-DOWN PROTEOMIC STUDY OF PANCREATIC JUICE

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Pancreatic juice (PJ) is an excellent source of proteins that are secreted directly from the pancreatic duct, where pancreatic cancers generally arise. Cancer cells are often shed into the ductal lumen, making PJ a rich source of cancer-specific proteins. PJ is a precious source for searching biologically significant proteins associated with pancreatitis or pancreatic cancer. Pancreatitis, an inflammatory condition of the pancreas, often has in common many molecular features with pancreatic cancer and biomarkers candidate present in pancreatic cancer frequently occur in the setting of pancreatitis [1]. We used a top-down proteomic approach for the analysis of PJ using liquid chromatography tandem mass spectrometry (HPLC-ESI-MS/MS). PJ samples from 7 patients with chronic pancreatitis, 1 neuroendocrine tumor (NET) and 1 pancreatic cancer (PC) were collected during diagnostic endoscopic retrograde cholangio-pancreatography. Immediately after collection an acidic solution of 0.2% aqueous trifluoroacetic acid was added to PJ in 1:1 v:v ratio. The solution was centrifuged at 10,000 g for 5 min and the supernatant immediately stored at -80 °C. 100 ul aliquots of the supernatant solution were directly injected into the HPLC-ESI-MS apparatus. Our study analyzed the proteome of pancreatitis juice in comparison with that of pancreatic cancer. Until now we were able to identify in the HPLC-ESI-MS profile the pancreatic trypsin inhibitor, phospholipase A2, cholecystokinin, carboxypeptidase A1, A2, B1, amylase, chymotrypsinogen, trypsinogen IV, elastase IIA, lysozyme, α-fetoprotein and thymosin β4. Preliminary results show differential proteins expression related to the specific pathological condition. The complete characterization of PJ is in progress in order to identify specific pancreatitis-associated proteins and to discover potential biomarkers of pancreatic cancer.

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DEVELOPMENT OF MRM MASS SPECTROMETRY BASED METHODS FOR VALIDATION AND SEMI-QUANTITATIVE EVALUATION OF CLINICALLY RELEVANT SERUM BIOMARKER IN PROSTATE CANCER

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Prostate cancer (PCa) is the most common cancer diagnosis and the second most common cause of cancer-related deaths in men. Currently, serum prostate-specific antigen is the only molecular biomarker widely used in the diagnosis and management of patients with PCa. However, it cannot distinguish indolent from aggressive disease nor can it be used to distinguish between different Gleason grades of disease. Multiple reaction monitoring (MRM) is a mass spectrometry strategy of sufficient reproducibility, sensitivity and selectivity to undertake quantitative measurement of panels of potential protein biomarkers. We have used our previous proteomic discovery data and literature analysis to establish a panel of 53 potential PCa serum protein biomarkers and here the results from the development of MRM measurements of these proteins are described. Proteotypic peptides and potential MS/MS fragments (transitions) for each protein were identified using SpectumMill (Agilent Technologies). Subsequently, the ability to identify each proteotypic peptide in serum depleted of abundant proteins was investigated by Q-Tof MS/MS and semi-quantitative measurement of peptide abundance in a QQQ MS was undertaken. Using this approach we have sought to semi-quantitatively measure specific potential PCa biomarkers in serum samples from four cohorts of patients (n=10 for each group): Gleason grade 5(G5), Gleason grade 7 (G7) organ-confined disease, G7 non organ-confined disease and age-matched controls and establish their correlation with different Gleason grade (G) disease and/or extracapsular extension.

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SCLERODERMA LUNG DISEASE AND β-THYMOSINS: BRONCHOALVEOLAR LAVAGE FLUID BIOMARKERS

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The role of β-thymosins ($T\beta_4$, $T\beta_4$ sulfoxide ($sT\beta_4$), $T\beta_{10}$) in cytoscheleton rearrangement, angiogenesis, fibrosis and reparative process suggests a possible involvement of these peptides in the pathogenesis of systemic sclerosis (SSc). The aims of the study were to demonstrate the presence of $T\beta_4$, $sT\beta_4$ and $T\beta_{10}$ in bronchoalveolar lavage fluid (BALF) of SSc patients with interstitial lung disease (ILD) and to correlate their levels with the biologic, functional and radiological parameters of the lung involvement and to the progression of ILD.

$T\beta_4$, $sT\beta_4$, $T\beta_{10}$ were determined by HPLC-ESI-MS in BALF samples of 46 SSc patients with ILD and of 15 control subjects.

$T\beta_4$ levels were significantly higher in SSc patients than in controls. Nevertheless, lower BALF Tβ4 levels were found in the SSc patients with significantly worsening in the alveolar score on HRCT after one-year follow-up. Higher $sT\beta_4$ BALF levels correlated with the smoking habits, the presence of alveolitis, the BALF neutrophil percentage count, an inverted BALF CD4/CD8 ratio, and a lower forced vital capacity. $T\beta_{10}$ levels directly correlated with the BALF lymphocyte percentage count and inversely with the carbon monoxide diffusing capacity.

We described for the first time the presence of $T\beta_4$, $sT\beta_4$ and $T\beta_{10}$ in human BALF. Our results suggest the possible involvement of these paracrine factors in the pathogenesis of scleroderma lung disease. As described in other studies, $T\beta_4$ seems to have a protective role against tissue damage even in SSc lung disease and its oxidation product seems to mirror the presence of an inflammatory condition.

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SALIVARY IMMUNOPEPTIDES IN PRIMARY AND SECONDARY SJÖGREN'S SYNDROME PATIENTS.

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We have previously described that higher α-defensin1 levels and the presence of β-defensin 2 in primary Sjögren's syndrome (pSS) saliva could be markers of the disease [1]. The aim of the present study was to investigate the presence and the levels of salivary defensins and thymosins in patients with secondary SS (sSS) associated to systemic sclerosis (sSS/SSc), systemic lupus erythematosus (sSS/SLE) and rheumatoid arthritis (sSS/RA) versus SSc, SLE and RA patients without SS and versus pSS and healthy controls.

Saliva specimens of 9 pSS, 7 sSS/SSc, 7 sSS/SLE, 7 sSS/RA, 7 SSc, 7 SLE, 7 RA patients and 10 healthy subjects were analysed by HPLC-ESI-MS. α-defensins 1-4 and β-defensins 1-2, thymosin β4 (Tβ4), thymosin β4 sulfoxide (sTβ4) and thymosin β10 (Tβ10)were investigated. α-defensins were significantly higher in pSS and sSS/SSc saliva respect to controls.

β-defensin 2 was found in 66.7% of pSS, 85.7% of sSS/SSc, 28.6% of sSS/SLE, 57.1% of sSS/RA, and in none of the healthy controls and patients without associated SS. Tβ4 levels were significantly higher in pSS compared to other subgroups.Tβ10 and sTβ4 were found in 66.7% and 44.4% of pSS and in 42.9% and 42.9% of sSS/SSc, respectively.

The results show that higher salivary α-defensin 1 and Tβ4 levels characterized pSS patients. Moreover, we found that the presence of β-defensin 2 can be considered a biomarker of primary and secondary Sjögren's syndrome.Tβ10 and sTβ4 seem to characterize pSS and sSS/SSc saliva.

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PROTEIN DIFFERENCES BETWEEN PATIENTS NORMOZOOSPERMIC AND ASTHENOZOOSPERMIC

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The molecular basis of asthenozoospermia, a potential cause of male infertility due to reduced sperm motility, is not yet fully understood and it is a very common finding in infertile patients.

A normal ejaculate should contain at least 50% of motile sperm and this parameter must be estimated, after semen liquefaction, through a spermiogram according to published recommendations (WHO 1999).

For proteomic analysis, a single ejaculate was processed from each participant after at least 3 days, but no longer than 5 days, of sexual abstinence. The seminal plasma and other potential contaminants cells present in semen were removed through centrifugation in 50% and 100% Percoll step gradient.

Spermatozoa from the pellet were solubilized in lysis buffer. The samples were then incubated for 1 h at room temperature to allow solubilization, and finally centrifuged to eliminate potentially insolubilized debris. The solubilized protein, was placed in the rehydration with linear strips (IPG pH 4–7). Below it is processed to SDS-PAGE. After electrophoresis, the gels were stained with Silver Stain. A comparison of the gel allowed us to pick the spots that now are being characterized by mass spectrometry (MALDI TOF-TOF).

In conclusion, the proteomic approach is important to identify proteins with altered amounts in the sperm cells of asthenozoospermic patients, opening the door to further identification of the mechanisms involved in asthenozoospermia and to their potential assessment as novel biomarkers of infertility.

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1.59

IDENTIFICATION OF CYTOSKELETAL PROTEINS MONOCLONAL ANTIBODIES RECOGNIZED BY CSF114(glc), THE SYNTHETIC PROBE OF MULTIPLE SCLEROSIS

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CSF114(Glc) is a simple, reliable, and efficient tool, capable of detecting autoantibodies in a statistically significant number of Multiple Sclerosis patients.[1, 2, 3] The corresponding native antigen(s) possibly triggering anti-CSF114(Glc) antibodies have not been yet identified and characterised. In this study, we demonstrated the ability of CSF114(Glc) to specifically recognize monoclonal IgGs antibodies of cytoskeletal proteins, identified by an immunoproteomic approach. Frozen rat brain was homogenized and solubilized in tritonX-100. Solubilized fractions were analysed in 12% SDS-PAGE. Extracted proteins were transferred onto Nitrocellulose membrane and western blots were performed using affinity purified anti-CSF114(Glc) IgGs. We constantly found three positive bands recognized by affinity purified anti-CSF114(Glc) IgGs at the positions of 130kDa, 98kDa, and 46kDa. Affinity purification of normal blood donors sera using the same CSF114(Glc) column did not recognize those bands.
Bands of interest from SDS-PAGE were excised and trypsin digested. Digested samples were analyzed by MALDI-TOF MS and MS/MS, PMF and MS/MS data were searched in swiss-prot database using MASCOT software. We identified 2',3'-cyclic-nucleotide 3' phosphodiesterase, brain specific Alpha actinin, and spectrin alpha chain (Alpha Fodrin) corresponding to positions 46kDa, 98kDa, and 130kDa respectively. To confirm our data, we purchased monoclonal antibodies to Alpha fodrin and CNPase (Abcam), and Alpha actinin (Sigma). All the monoclonal IgGs were used in BiaCore for binding analysis with CSF114(Glc) coated chips. We found that anti-Alpha actinin and anti-Alpha fodrin were able to bind to CSF114(Glc) while anti-CNPase IgGs were not able to bind to CSF114(Glc) at the same concentration.

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PROTEIN EXPRESSION PROFILES IN BRONCHOALVEOLAR LAVAGE FLUID OF PULMONARY LANGERHANS-CELL HISTIOCYTOSIS, NO-SMOKING AND SMOKING HEALTH SUBJECTS.

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Pulmonary Langerhans cell Histiocytosis (PLCH) is a rare interstitial lung disease with unknown etiopathogenesis. Cigarettes smoke is thought to be the principal cause in inducing inflammatory process, which is related to the formation of lung nodules in the distal bronchioles. To better understand the possible pathogenetic mechanisms of this disease, a proteomic analysis was carry out. Bronchoalveolar lavage samples from PLCH patiens and no-smoking- and smoking-controls were compared by 2DE and Image Master Patinum 7.0 software. The statistical analysis was performed by one-way ANOVA and Tukey's testes. Sixty quantitative and 10 qualitative differences were found out and several of them were identified by MALDI-TOF MS and LC MS/MS. Alpha-1 antitrypsin ($p<0.05$), Apolipoprotein AI ($p<0.01$), and Annexin A3 ($p<0.02$) were detected among the PLCH up-regulated proteins. Alpha-1 antitrypsin is involved in the protection of the lower respiratory tract against proteolytic destruction carried out by leukocyte elastase during inflammation. Interestingly, Apolipoprotein AI and Annexin A3 were also described up-regulated in lung cancer. On the other hand, Plastin-2 ($p<0.01$) and Polymeric immunoglobulin receptor ($p<0.02$) are down-regulated in PLCH. Plastin-2 operate in the activation of T-cells while Polymeric immunoglobulin receptor is a central player in mucosal immunity. In conclusion the proteomic analysis of BALF from patients with PLCH could represent a valid approach to better define the pathogenetic mechanisms involved in this rare diffuse lung disease.

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KINETICS OF DIFFERENT PROCESSES IN HUMAN ANP AMYLOID FORMATION

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Atrial Natriuretic Peptide (ANP), a soluble circulating protein, can be amyloidogenic and features ANP-containing amyloid deposits are found primarily in the heart [1]. The cause for this transition from soluble to insoluble protein in Isolated Atrial Amyloidosis (IAA) is yet to be determined, and specific structural features that might favour ANP fibrillogenesis have not yet been identified. The molecular basis of ANP aggregation is thus relevant for modelling the amyloidogenesis process as well as for improving delivery systems used for amyloidosis treatments. Consequently, the precise characterization of *in vitro* fibril deposits might provide insight the true biochemical nature of deposited ANP. In this study, we provide detailed analyses of both the soluble and deposited forms of ANP in different solution conditions. The structure and the morphogenesis of aggregates have been monitored by using different experimental techniques: Transmission electron microscopy, spectrophotometric Congo Red assay, thioflavin T fluorescence, FTIR spectroscopy. ANP aggregation displays a wide variety of morphologies, from small oligomeric filaments to voluminous floccules, and therefore different specific processes are likely to be intertwined in the overall aggregation. Fibrillar aggregates grow following a concentration dependent heterogeneous coagulation mechanism, including both protofibril elongation and lateral thickening until formation of strictly interconnected amyloid material.

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PROTEOMICS INVESTIGATION OF HUMAN PLATELETS IN HEALTHY DONORS AND CYSTIC FIBROSIS PATIENT BY SHOTGUN NUPLC-MSE AND 2DE: A COMPARATIVE STUDY

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Platelets are the smallest blood circulating cells involved in several pathophysiological events such as haemostasis, wound repair, inflammation and cardiovascular disease. Although platelets are anucleated particles and inherit their protein content from megakaryocytes, they can undergo translation events and synthesize selected proteins. Thus, the analysis of the platelet proteoma may provide significant information on the role that platelets may play in human pathology.
Cystic fibrosis (CF) is a genetic disease evoked by mutations in the CFTR gene, located in the long arm of chromosome 7 and coding for a protein with ion channel activity. Inflammatory lung disease is the primary cause of morbidity and mortality in CF, nonetheless mechanisms of CF inflammation are still incompletely known. Enhanced platelet activation in vivo and ex vivo has been documented in CF patients which display also increased circulating platelet/PMN and platelet/monocyte complexes, suggesting that platelets may be involved in CF inflammation.
In this work, we pursued a comparative proteomic analysis of platelets obtained from healthy donors and CF patient, employing two different, but complementary approaches, namely the classic 2-DE in association with mass analysis and nanoscale ultra-performance LC-MSE. We compared these techniques for sensibility, accuracy and data reproducibility. We consistently observed that the combination of these techniques gives a more detailed characterization of the platelet proteome and identified, a restricted group of protein differentially expressed in CF platelets.
A comparative analysis of the Gene Ontology was performed to evaluate the overall molecular function and the potential pathophysiological relevance of these proteins within the context of CF inflammation

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MALDI IMAGING - A NEW TOOL FOR MAPPING MULTIPLE SCLEROSIS BRAIN LESIONS

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Remyelination of Multiple Sclerosis (MS) brain lesions is an important mechanism to prevent chronic disability. Proteins including nerve growth factor, neurothrophic factors and others that affect myelin stability and have antioxidant functions play an important role by either promoting or inhibiting remyelination. The mechanism of remyelination and which proteins are involved in this process remain elusive. Recently, advances in the visualization of the distribution of proteins and peptides in tissue have been made by imaging mass spectrometry (IMS) using MALDI-MS technology[1]. We have employed the MALDI-IMS with the new laser technology Smartbeam™ for the analysis of human MS brain lesions to gain information about protein and peptide composition of normal appearing white matter and lesions of MS brain tissue presenting different grades of remyelination. For this purpose we measured the entire tissue slide by acquiring protein and peptide signals in the 2000 to 25000 Da mass range. The statistical analyses of the MALDI spectra, performed by an unsupervised hierarchical clustering[2] based on the similarity of the spectra and by the principle component analysis (PCA), permitted the generation of molecular weight tissue images. These spatial distributions of specific masses or groups of masses generated and defined regions inside the tissue slide which are in agreement with those obtained from the histological analysis. Detection of tissue structure not detectable by histopatology is gained by MALDI-IMS.
The application of MALDI-IMS has yielded in the mapping of molecular components leading to sufficient or partial remyelination and being not related exclusively to Myelin Basic Protein.

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PROTEIN EXPRESSION PROFILE OF PLASMEPSIN IV KNOCK-OUT *PLASMODIUM BERGHEI* PARASITE AND IMMUNOREACTIVE PROTEINS IDENTIFICATION

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Malaria still represents in vast regions of the developing world particularly sub-Saharan Africa one of the major causes of morbidity and mortality amongst young children. The disease is caused by blood stage parasites that invade and multiply within host erythrocytes. Decades of efforts aimed at developing either subunit or attenuated live organism vaccines to malaria blood stage parasites have so far been inconclusive. In the rodent malaria parasite *Plasmodium berghei,* the gene disruption of plasmepsin IV, a food vacuole aspartic protease involved in haemoglobin digestion, unexpectedly generated virulence-attenuated parasites that elicited a strong and long lasting protective immunity [1]. Proteome of this transgenic parasite was obtained by 2D electrophoresis and compared to that of wt *P. berghei*. Moreover, using serum collected from protected animals we could visualise in *P. berghei* proteome a number of antigen with different immunoreactivity. Serum from protected mice was then used to immunoprecipitate antigens for their mass spectrometry identification. The analysis identified several proteins some of which are associated to the surface of parasites thus representing good targets for vaccine development.

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URINE AND SERUM PROTEIN PROFILE MODIFICATIONS OF RENAL CELL CARCINOMA EVALUATED BY CLINPROT TECHNIQUE

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Renal Cell Carcinoma (RCC) is one of the major cause of cancer death and is radio- and chemo-resistant. Detection of RCC in its early stage and of benign renal masses is increased as a consequence of the incremental use of imaging procedures. However the nature of some cystic and solid renal lesions cannot be confidently diagnosed by imaging techniques. Therefore it appears important to find biomarkers for the early detection, for differential diagnosis of RCC from benign renal lesions, for prognosis, and follow-up. Results obtained in retrospective studies have shown that multivariate predictive models combining existing tumor markers improve cancer detection. To this purpose we investigated the possibility of using an innovative approach, known as ClinProt, based on an off-line fractionation of the proteome of biological fluids using magnetic beads with activated surface followed by MALDI-TOF MS analysis in order to find possible diagnostic markers that can better distinguish healthy subjects from RCC patients. Up to now we applied ClinProt technique both on RCC urine [1] (n=39) and serum [2] samples (n=33) in order to search a diagnostic test with high specificity and sensitivity. Our results suggest that a proteomic approach based on magnetic beads represents an useful method to discover possible clinical biomarkers. Moreover we demonstrate the capability of selected signals to differentiate RCC patients from normal subjects. Therefore, the selected cluster of peaks represents a promising diagnostic tool for RCC independently from the knowledge of peptide structures giving origin to the signals.

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CHARACTERIZATION OF TWO ISOFORMS OF HUMAN SPRR3 FROM HUMAN PRETERM NEWBORN SALIVA, AUTOPTIC FETAL ORAL MUCOSA AND PAROTID GLAND SAMPLES

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RP-HPLC-ESI-MS profile of saliva samples from human preterm newborns showed a peak in the elution range 26.6-27.6 min that has never been detected before in adult saliva [1]. Deconvolution of ESI-MS spectra revealed the presence of two proteins with average molecular mass (Mav) values 17239 ± 3 Da and 18065 ± 3 Da in 9 samples, with Mav 17239 ± 3 Da in 4 samples and Mav 18065 ± 3 Da in 2 samples. MALDI-TOF-MS and MS/MS analysis of tryptic digests allowed identifying the two proteins as small proline-rich protein 3 (SPRR3; Swiss-Prot code Q9UBC9, A5YKK8) [2], previously described in two isoforms differing for an octapeptide repeat (GCTKVPEP) and the substitution Leu148→Val [3]. cDNA amplification of RNA extracts from oral mucosa and parotid gland samples obtained at fetal autopsy provided two nucleotide sequences in agreement with those reported in the literature.
The difference between experimental and theoretical Mav values may be explained with the removal of the initiator methionine (-131 Da) and the presence of an acetyl group, the latter confirmed by incubation in the presence of 25% TFA at 55 °C for 60 min. These two post translational modifications have not been previously described for these proteins. Immunohistochemistry experiments and/or signal peptide sequencing will allow clarifying if the proteins found in saliva originate from cellular shedding of the epithelium and/or gland secretion.

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1.67

STUDY OF URINARY EXOSOMES COMPOSITION IN AN ANIMAL MODEL OF DIABETIC NEPHROPATY

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Diabetic nephropathy (DN) is a common diabetic complication, associated with alterations in the expression of several renal proteins. The identification of early biomarkers of diabetic nephropathy might be useful to monitor the disease progression. We have recently observed differences in renal protein expression profiles in a rat model of type 1 diabetes (streptozotocin induced), compared with control and insulin-treated diabetic rats. Now we are investigating the protein composition of urinary exosomes, nanovescicles normally released into urines from all nephron segments, since exosome analysis represent a potential approach for urinary biomarkers discovery.

To this aim, diabetes was induced in 10 Sprague Dawley rats by streptozotocin injection. Control rats (n=4) underwent only buffer injection. Out of 10 diabetic rats, 4 were chronically treated with insulin. After 3 months, 24 hours urines were collected. Urinary exosomes were isolated by ultracentrifugation, and protein analysis was performed by SDS-PAGE/CBB staining and by 16-BAC/SDS-PAGE/SyproRuby staining.

Protein profiles of the three rat groups show differences, mainly at low molecular weights. Some differential bands/spots were excised and identified by LC-MS/MS, and resulted to be related to the Major Urinary Proteins, known as MUPs, belonging to the lipocalin family. These secreted proteins are involved in the binding and release of low molecular weight pheromones or other hydrophobic molecules but MUPs themselves may also function as chemosignalling molecules. Moreover MUPs regulate glucose and lipid metabolism suggesting an involvement in hyperglycemia, insulin resistance and/or glucose intolerance in diabetes.

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1.68

CONTRIBUTION OF URINE PROTEOME ANALYSIS FOR NON-INVASIVE DIFFERENTIAL DIAGNOSIS OF DIABETIC NEPHROPATHY

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The increasing incidence of diabetes related kidney injury asks for the development of new and non invasive tools for achieving early and differential diagnosis of diabetic nephropathy (DN). We interrogated urine proteomic profiles with the aim of isolating a set of biomarkers able to reliably identify biopsy-proven DN and to establish a stringent correlation with the different patterns of renal injury. SELDI-TOF/MS technology coupled with supervised statistical methods was used to differentiate type 2 diabetic patients with biopsy-proven DN from patients without glomerular filtration rate (GFR) impairment namely normoalbuminuric (NAD) or microalbuminuric (MICRO) patients as well as from subjects with non diabetic chronic kidney diseases (ND-CKD). Ten µg urine protein from 169 subjects [20 healthy subjects (HS), 38 diabetic patients without renal impairment and 111 patients with biopsy-proven nephropathy (54 DN and 57 ND-CKD)] were run by CM10 proteinchip array and analysed by supervised methods (CART analysis). The classificatory model correctly classified 87.5% NAD, 75% MICRO and 100% DN patients. Subsequently, it differentiated DN from ND-CKD with 78% sensitivity and 87.5% specificity, respectively. Among the differently excreted DN mass peaks two were identified as ß2-Microglobulin and Ubiquitin and their significant differential excretion in urine of DN patients were further validated by ELISA and immunoblotting respectively. **CONCLUSIONS** Our data suggest the presence of a specific urine proteomic signature able to reliably identify type 2 diabetic patients with diabetic glomerulosclerosis. The identification and validation of ß2-Microglobulin and Ubiquitin as DN differentially excreted urine proteins stresses the usefulness of proteomic based approach for the identification of new reliable DN biomarkers potentially involved into the pathogenetic mechanisms of renal damage.

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1.69

IDENTIFICATION OF FERTILITY BIOMARKERS BY 2D-SDS PAGE AND MALDI - TOF / TOF

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Human seminal plasma contains a large number of proteins required for the normal physiology of spermatozoa. It contains proteins secreted from the testis, epididymis and glands reflecting its functionality. To provide informations about the physiological mechanisms of male fertility we performed proteomic study on human seminal plasma by 2D SDS PAGE and MS/MS analysis.

The purpose of this study was to identify genes potentially involved in male infertility through the analysis of protein profiles of fertil subjects and patients with nonobstructive azoospemia.

The semen collected according to the criteria recommended by WHO, was centrifuged to remove cellular debris and the supernatant was used as seminal plasma and stored at - 80 ° C until analysis.

An aliquot of the sample was precipitated by ProteoExtract Protein precipitation kit and resuspended in rehydration buffer (7M urea, 2M thiourea, 4% chaps). After IEF on IPG-strip pH 4-7, proteins from each strip were separated on 4-12 % vertical polyacrylamide precast gels and stained with Colloidal Blue Coomassie and Silver Stain.

The comparison of the gel revealed the presence of numerous spots differentially expressed. These spot were excised, digested by trypsin and characterized by MALDI TOF/TOF MS. Coomassie blue detection was considered a good quantitative indication for spot excision.

The protein identification by mass spectrometry analysis and search in database allowed identification of human PPAP, SEMG1, SEMG2, CXB1, KLK3 and PIP.

Subsequent investigations are needed to clarify the role of these proteins in fertility and their function in spermatogenesis.

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1.70

DIFFERENTIAL PROTEOMICS OF RCC URINARY EXOSOME: AN UPDATE

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Clear cell renal carcinomas (RCC) is representing about 3% of all kidney cancers. No biomarkers for diagnosis of RCC or for post-surgery monitoring are yet available. Urinary exosomes have been shown to derive from every epithelial cell type facing the urinary space. For this reason they are considered an important source of urinary proteins and a promising starting material for biomarker discovery. Aim of the work is to study protein composition of exosomes isolated from RCC patient urines compared with control ones in order to detect differences, potential tumor marker. Exosomes were isolated by ultracentrifugation from about 30 ml of urines collected from 28 control healthy subjects and 41 patients (29 clear cell RCC, 6 malignant non-clear cell and 6 benign). Protein profiling was addressed by SDS-PAGE and 16-BAC/SDS-PAGE, followed by CBB staining or WB with antibodies against specific membrane proteins. Results show that the proteomic profiles of urinary RCC and control exosomes are different and quite reproducible. Some relevant membrane proteins (AQP-1, Pgp, EMMPRIN, CA9, MMP9) display differential amount in RCC patient urine exosomes. Moreover the analysis of exosomes purified from follow-up urines of RCC patients show that after two years from surgery protein levels change again and are similar to controls. In conclusion the results suggest that the process of protein compartmentalization into exosomes is selective and that urinary exosomes show potential clinical usefulness.

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1.71

PROTEOMIC PROFILE OF CD24$^+$ CD133$^+$ RENAL MULTIPOTENT PROGENITORS (RMP)

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A population of Renal Multipotent Progenitors (RMP) in adult human kidneys CD24$^+$ and CD133$^+$ is able to repair injured renal tissue of SCID mice with acute tubular necrosis.

In our laboratory, we defined the proteomic profile of a well fenotipically and functionally characterized Renal Multipotent Progenitors. RMP cell lines were isolated from normal kidneys of 30 patients undergoing nephrectomy for renal carcinoma. At first passage after isolation, flow cytometric analysis showed that the cell pool was CD24$^+$ (60,0%) and CD133$^+$ (25,4%), while 20.6% of pool cell co-expressed both markers. Total protein extracts of two cell line were analyzed by 2D-PAGE analysis combined to nanoHPLC-ESI-ion trap-MS/MS and MALDI-TOF-MS analysis. An average of about 1080 spots, characterized by their pI and MW, were detected in the silver stained gels of total protein extract. The protein spots identified are involved in cellular cytoskeleton (28,6%), stress response (23,8%), cellular metabolism (14,3%), cell proliferation and differentiation (9,5%). Now we are performing the differential proteomic profiling of CD24$^+$CD133$^+$RMP treated with rapamycin to study the effects of mTOR inhibition on RMP phenotype. Rapamycin may affect the RMP biology via the modulation of HIF1alpha/VHL pathway, which regulates downstream the CXCR4 and SDF-1 genes, both required for therapeutic homing of RMP *in vivo*. Rapamycin treatment increased CD133$^+$CD24$^+$ cell population, while it decreased RMP proliferation and chemokinesis, moreover it didn't affect cell viability.

The study of proteome of RMP cell line allowed the identification/discovery of molecules for a better comprehension of RMP biology and mechanisms of renal repair.

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1.72

PROTEOMIC DETECTION OF S100 PROTEINS IN BREAST CANCER TISSUES

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One class of proteins that is emerging as a potentially important group of markers in cancer development and progression is the S100 family. The associations between S100 family members and tumor can be explained for at least three different reasons: firstly, the region of human chromosome 1q21 where most of S100 genes are clustered is prone to genomic rearrangements, suggesting a link with tumor formation and metastasis. Secondly, several S100 members show altered expression levels in cancer cells compared to normal cells, and are differentially expressed in various malignancies, depending on the type and stage of cancer. Finally, a number of S100 proteins have been shown to interact with and to regulate various proteins involved in cancer and exert different effects on p53 activity.

The aim of the present work was to investigate the proteomic distribution of the S100 family member within a cohort of 100 breast cancer patients. Preliminary results have shown that the majority of S100 proteins were present at very low levels, if not absent, in the non-tumoral tissues adjacent to the primary tumor. The proteomic screening of the 100 patients showed that some of the S100 protein members were ubiquitously expressed in almost all patients, while others appeared more sporadic. Most, if not all, of the detected S100 members appeared reciprocally correlated. More interestingly, patients which developed distant metastases after a three years follow-up, showed a general tendency of higher S100 protein expression, compared to the disease-free group.

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1.73

DEVELOPMENT OF A RANKING METHOD FOR THE IDENTIFICATION OF POSSIBLE BIOMARKERS IN PROTEOMICS BASED ON PRINCIPAL COMPONENT ANALYSIS AND VARIABLE SELECTION

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The identification of pools of biomarkers is one of the leading research areas in proteomics. When biomarkers have to be searched for in spot volume datasets produced by 2D gel-electrophoresis, problems may arise related to the large number of spots present in each map and the small number of samples available for each class (control/pathological). In such cases multivariate methods are usually exploited together with variable selection procedures, to provide a set of possible biomarkers: they are however usually aimed to the selection of the smallest set of variables (spots) providing the best performance in prediction. This approach, known as the "principle of parsimony", seems not to be suitable in proteomics since the most discriminating spots are usually related to general responses to inflammatory events rather than to the pathology under investigation. Moreover, the principle of parsimony identifies as differentially expressed only a subset of these most discriminating spots, i.e. the smallest possible set. We propose here a ranking and classification method, "Ranking-PCA", based on Principal Component Analysis and variable selection in forward search: the method selects one variable at a time as the one providing the best separation of the two classes investigated in the space given by the relevant PCs. The method was applied to artificial datasets and real case-studies: in all cases, Ranking-PCA exhaustively identified all the potential biomarkers and provided reliable and robust results.

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1.74

PLASMA PROTEOME: A BETTER INSIGHT INTO TANGIER DISEASE

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Familial HDL deficiencies are a heterogeneous group of monogenic disorders characterized by low plasma levels of HDL cholesterol (HDL-C) [1]. Among them, Tangier disease (TD) is a rare autosomal recessive disorder characterized by a marked deficiency of HDL-C in plasma caused by mutations in the adenotriphosphate-binding cassette transporter-1 (ABCA1) gene. Mutations in ABCA1 lead to a defect in cellular cholesterol removal causing the accumulation of cholesterol esters throughout the body [2]. Individuals with TD are unable to eliminate cholesterol from cells which is then accumulated in the macrophage rich tissues [2].
In the 2009 TD was diagnosed to a 37-year-old man [3] at Fondazione G. Monasterio-CNR, Pisa. For a better understanding of disease, we performed a proteomic study of plasma samples from homozygous TD patient and from the heterozygous father. Crude plasma samples were enzymatically digested and extensively analysed by an HPLC-MALDI approach. The peptide fractionation time and conditions were optimised and the method performance checked in terms of protein identifications. Additionally, this method enabled us to analyse profiles of differentially expressed proteins in patients versus control healthy volunteers. We identified more than 2000 plasma proteins with a good sensitivity for less represented proteins such as Prothrombin (1.2 pg/ml) [4]. A number of differentially modulated proteins were observed and their statistical significance assessed. Clinical diagnostic nephelometric assays, performed on some among the identified proteins, displayed an actual modulation of serum concentration between healthy controls and patients specimens confirming the differences observed in proteomic comparative analysis.

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1.75

MULTIPLEX WESTERN BLOTTING BY CYDYE3 AND CYDYE 5 LABELLED CONJUGATES

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Background: Recently, secondary antibodies conjugated with cyanine CyDye reagents have been produced for western blot immunodetection without addition of enhancer to improve sensitivity of weak signals in multiplex assay, and reduce membrane replicates. In this work we applied the new reagents to confirm and quantify proteins differentially expressed in renal cell carcinoma (RCC) [1].

Methods: Two protein pools obtained from normal renal tissue and RCC were separated by SDS-PAGE, transferred into Hybond PVDF membranes, and probed with anti-Hsp27 mouse antibody and anti-mitofilin rabbit antibody. The Hsp27- and mitofilin-antibody complexes were resolved by incubation with anti-mouse-CyDye 3 and anti-rabbit-CyDye 5, visualised using Typhoon 9400, then stained with Sypro Ruby Blot. Each pool was performed in triplicate on a single gel for 4 consecutive days.

Results: We calculated the inter-assay Hsp27 coefficient of variation (CV) (range 11.5% -22.78%) in normal tissue and (8.34% to 39.62%) in RCC. Mitofilin CV ranges were 0.98% - 25.31 in normal tissue and 5.67 - 21.85% in RCC. The intra-assay Hsp27 CV were 35.75% and 32.64%, respectively, from normal tissue and RCC, while that of mitofilin were 31.57% and 39.51%. The differences of protein profile between normal and RCC tissues ranged from 1.02 to 1.77 for Hsp27, and from 1.04 to 1.91 for mitofilin.

Conclusions: Secondary antibodies labelled with the two different CyDye reagents allowed the simultaneous detection and quantitation of two diverse proteins without membrane stripping and re-probing. This ensures low cross-reactivity and variability in protein quantitation, and reduces processing time.

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1.76

IDENTIFICATION OF α DEFENSINS 1-2 IN SALIVARY STONES OF SUBMANDIBULAR GLAND

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Sialolithiasis is the most common disease of major salivary glands with an incidence of 1.2%. Several studies were carried out to determine causes and processes of formation of salivary stones, but they have not provided clear results. Sialoliths could derive, as a secondary event, from the chronic obstructive inflammation of salivary glands [1], and the composition of calculi varied in the content of mineral and organix matrix. Hydroxyapatite is the most common mineral in sialoliths but other minerals could also be present, while the organic matrix is mainly constituted of lipids and glycoproteins.

At our knowledge no proteomic studies were done on salivary gland calculi. The aim of this study was to investigate the peptides composition of submandibular salivary gland stones by using a proteomic top-down approach. Mineral analysis of the stones was also performed.

The protein extract of stones was analyzed by using MALDI-TOF instrument in the 1000-7000 peptide range mass analysis. Several small peptides were detected. Among them the presence of α-defensins 1 and 2 was evidenced. The identity of the two peptides were confirmed by capillary LC-ESI-LTQ Orbitrap experiments.

A previous study reported the presence of this peptide also in calcium oxalate renal stones speculating its possible role in nucleation of stone genesis [2]. α-defensins are small cationic peptides. They have a broad antimicrobial activity and, in addiction participate in multiple aspects of innate immunity and inflammation. The functions of these peptides could justify their involvement in the mechanism of salivary gland stone formation.

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1.77

IDENTIFICATION OF PREDICTIVE BIOMARKERS IN ADVANCED PANCREATIC CANCER PATIENTS UNDERGOING GEMCITABINE-OXALIPLATIN CHEMOTHERAPY

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The aim of our research program is the identification, through proteomic analysis of plasma proteins, of potential response markers to chemotherapy by highlighting differences in protein profile between responsive and non-responsive patients.

In an exploratory analysis we enrolled 6 patients with histologic diagnosis of locally advanced or metastatic pancreatic adenocarcinoma who received bi-weekly gemcitabine 1000 mg / sqm on day 1 and oxaliplatin 85 mg/sqm on day 2 [1] as first-line chemotherapy. The plasma samples were collected before chemotherapy (day 1 of the first course) and 48 hours after the end of the same treatment, according to standards of the Human Proteome Organization (HUPO).

Plasma proteomic analysis, performed by 2D-PAGE coupled to liquid chromatography tandem mass spectrometry (LC-MS/MS), allowed the identification, in the post-treatment samples, of two proteins, clusterin and alpha-1-microglobulin, which, in the light of their biological function, might very well correlate with treatment resistance; the former by inhibiting cancer cells apoptosis induced by drugs [2], the latter by increasing the metabolism of radical oxygen species (ROS), that are associated with the antiproliferative activity of the drug [3]. To confirm these findings, we have analyzed clusterin and alpha-1-microglobulin on human pancreatic adenocarcinoma cell lines BXPC-3 and PSN-1 by Western blot and found that tumor cell exposure to gemcitabine and oxaliplatin resulted in overexpression of the two proteins. We are presently scaling-up the prospective series of pancreatic cancer patients for assessing the true value of clusterin and alpha-1-microglobulin as predictive biomarkers, on the basis of the proof of principle findings reported here.

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1.78

PEPTIDOMIC ANALYSIS OF INDUCED SPUTUM OF ASTHMATIC PATIENTS BY MSB-MALDI-TOF-MS PLATFORM

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Asthma is one of the most common diseases of the airways in the Western industrialized world, characterized by intermittent airflow obstruction, bronchial hyperresponsiveness and flogistic infiltrate. Surprisingly, only a few proteomics studies on the ever-increasing global asthma problem have been published so far.

Traditional methods employed for studying inflammatory profile associated with airway diseases (bronchial biopsy, bronchial brushing and bronchoalveolar lavage) are too invasive to be easily applied in research study. Recently, non-invasive techniques, such as analysis of induced sputum, have been introduced. Induced sputum has the advantage that it is simple and safe to obtain, it is acceptable to patients and it allows to evaluate the inflammatory phenotype in asthmatic subjects.

Nevertheless, induced sputum is a complex biological fluid characterized by the presence of salts and abundant, high-molecular-weight and highly charged mucins that complicates its processing.

We are now investigating a new platform based on mesoporous silica beads (MSB) for induced sputum peptidomic profiling. Given the high surface area, mesoporous silicates offer the desired adsorptive capacity for harvesting low molecular weight peptides present in body fluids. Induced sputum samples are exposed to MSB and, then, captured molecular species are extracted and profiled by MALDI-TOF-MS.

MALDI-TOF mass spectra of induced sputum, collected from 9 patients and treated with MSB, reveal a significant enhancement in signal intensity as well as the number of peaks detected, especially in the range between 800 and 5000 Da.

These preliminary results encourages us to improve this platform to identify specific biomarkers associated with asthma.

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1.79

PLASMA GELSOLIN PROTEIN: A CANDIDATE BIOMARKER FOR HEPATITIS B-ASSOCIATED LIVER CIRRHOSIS IDENTIFIED BY PROTEOMIC APPROACH

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Despite the significant improvement in internal medicine and supportive therapy in recent years, liver fibrosis/cirrhosis remains a serious health issue in hepatitis B virus (HBV) infected patients [1,2]. Invasive liver biopsy is presently the best means of diagnosing cirrhosis, but it carries a significant risk and has well recognised limitations such as sampling error, hence the importance in developing early diagnosis biomarkers [3]. In recent years, plasma-based tests of liver cirrhosis have attracted more attention because plasma sample can be easily obtained from blood collection of patients. With this aim, we performed a pilot proteomic study to assess this as a strategy for plasma marker detection in patients suffering from HBV-associated liver cirrhosis. Plasma from eight chronic HBV-infection patients and from eight HBV-related cirrhotic patients were selected and proteome profiles were created by two-dimensional electrophoresis. The strategy included the use of ProteoMiner™ enrichment kit for the reduction of characteristic highly abundance proteins in plasma sample [4] (e.g. albumin and IgG) prior to proteomic analyses with the goal to improve detection of novel candidate markers. One reproducible spot was found to be completely repressed in plasma samples from cirrhotic patients and mass spectrometry analysis identified this a specific variant of the gelsolin actin-depolymerizing factor. Though further investigations are needed, especially in term of clinical validation, to our knowledge this is the first time that gelsolin is proposed as potential biomarker in HBV-related liver pathologies.

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1.80

IFN-B REVERSES THE LIPOPOLYSACCHARIDE-INDUCED PROTEOME MODIFICATIONS IN TREATED ASTROCYTES

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Interferon-beta (IFN-β) is a pleiotropic cytokine with a disease-modifying effect in patients with multiple sclerosis (MS). The exact mechanism by which IFN-β mediates its activity has been the object of study in several works [1]. However, its action on the central nervous system (CNS) is still debated and not completely elucidated. In the CNS, all three predominant cell types, neurons, oligodendrocytes and astrocytes, act as IFN-β producers and responders. In particular, astrocytes have a key role in the pathogenesis of multiple sclerosis, and are proposed as a possible target for immunotherapy. Our earlier study reported that astrocytes treated with IFN-β modified their biomechanical properties possibly due to changes in the expression of the proteins involved in cytoskeleton organization and other important physiological processes [2]. To gain insight into the mechanism underlying IFN-β action during inflammation, we stimulated astrocytes with LPS, a bacterial wall component used as a model for both in vitro and in vivo immunological stimulation of microglia and astrocytes. We showed that IFN-β reverses the effects of LPS on the proteome of astrocytes. To better examine this result, we performed a proteomic analysis of astrocytes treated with LPS or LPS plus IFN-β. Treatment with LPS caused increases both in a series of proteins mainly involved in cytoskeletal changes and in protein degradation, as well as protective enzymes like superoxide dismutase. IFN-β reverses LPS effects on astrocytes proteome, supporting its protective role during inflammatory insults.

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1.81

PROTEIN MODULATION IN MOUSE HEART AND MUSCLE UNDER ACUTE AND PROLONGED HYPOXIA

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Human heart diseases are characterized by an insufficient oxygen supply. The discovery of novel biomarkers specific for early, middle and late stages of heart diseases will be mandatory for diagnostic improvements. Proteomics could provide a clear picture of molecules involved in the onset/progression of diseases monitoring protein expression levels, post-translational modifications and degradation. In this study we employed hearts and muscles removed from mice under normoxic condition and exposed to constant hypoxia (8% O_2) for 48 hours (acute hypoxia) or 10 days (prolonged hypoxia).
Protein changes induced by hypobaric hypoxia in heart and muscles under acute and prolonged hypoxia were assessed by 2D-DIGE coupled with MALDI-ToF mass spectrometry. The modulated proteins were grouped in functional categories: cytoskeletal and myofibrillar proteins, chaperones, antioxidant enzymes, proteins involved in translation and degradation. These functional categories were differentially influenced in both hypoxic conditions compared to normoxia. As regard to metabolism, in particular in the heart, the acute hypoxia exposure indicated a decrement of some glycolytic enzymes while under prolonged hypoxia they were normalized or increased. By contrast, the expression levels of several aerobic enzymes were significantly decreased under prolonged hypoxia.
The second aim of this study was to identify and quantify S-nitrosylation targets in heart proteome since S-nitrosylation plays an important role in the cellular response to hypoxia. Normoxic protein extract was treated with GSNO as NO donor; S-nitrosylated molecules were detected using a "fluorescence switch" technique[1] based on a fluorescence derivatization of S-nitrosylated proteins and their separation and quantitation by 2-DE.

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1.82

TOP-DOWN PROTEOMIC ANALYSIS OF HUMAN CERVICAL MUCUS

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Human cervical mucus (HCM) is produced by cervical epithelial cells [1,2] and it is an important barrier for the upper genital tract against infection. Depending on the cyclical changes, it acts as either a hindrance or transport medium for spermatozoa and it is a relevant body fluid involved in human fertility [3,4]. Biochemical analysis of CM is difficult for the small amount of material, its heterogeneous composition and cyclical alterations of its rheological properties [5]. In the present study the throughput analytical technique of mass spectrometry was applied to the characterization of CM protein and peptide components by a top-down approach. Human cervical mucus was collected from the lumen of cervix from nineteen volunteers, eight women who got pregnant and eleven who did not get pregnant, at different periods of menstrual cycle. The samples were treated with 0.2% trifluoroacetic acid and the acidic soluble fraction analyzed by RP-HPLC-ESI ION-TRAP MS. By this approach it was possible up to now to identify the following components: α-defensins 1, 2, 3 and 4, secretory leukocyte peptidase inhibitor, lisozyme, cystatin A , S100 A8, S100 A9, S100 A12, cystatin B, and thymosin β4 and β10, together with a lot of small fragments of bigger proteins with potential biological activity. Detection frequency varied from 100% (α-defensins 1-3) to 9.1% (S100A12 and thymosin β10). This is the first report of the presence of thymosin β10 in human cervical mucus.

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1.83

SPECIE-SPECIFIC IGE RESPONSE TO MOSQUITO BITES: ANALYSIS OF ALLERGENS PATTERN FROM THE TWO SPECIES *AEDES ALBOPICTUS* AND *CULEX PIPIENS*.

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Allergic reactions to mosquito bites are very common and may cause severe local and systemic reactions including generalized urticaria and anaphylaxis. Since no mosquito salivary preparations, required for accurate diagnosis and immunotherapy are available, these reactions are under recognized and seldom treated with allergen immunotherapy.
Besides this, there was a rapid global spreading of the tiger mosquito *Aedes albopictus.* In Italy, this specie compete to *Culex pipiens*, the common mosquito, causing the onset of many allergic reactions. In this work we considered the reactivity of the serum from an allergic patient to the extract of salivary allergens from head and thorax of the two species *Aedes albopictus* and *Culex pipiens*. Animals were reared in laboratory; adult females were collected and stored at -80°C until used. Heads and thoraxes were then cut and pooled in micro-centrifuge tubes with water. They were pestled and centrifuged at 12,000xg for 10 minutes, then the supernatant was assayed for the total protein content and analyzed by SDS-PAGE and 2D- Electrophoresis. Western blot analysis was performed using the serum of a patient showing systemic reaction to the *Aedes albopictus* and negative to the commercially available Coated Allergens Particle (CAP) test for mosquitoes. Besides the presence of many shared allergens, these experiments showed specie-specific Ig-E response to *Aedes albopictus* proteins compared with *Culex pipiens* pattern. Based on these preliminary data we can hypothesize the application of this extract both in clinical uses and for *in vitro* investigation, in order to avoid false negative results in allergic subjects .

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2.1

A NEW SACI BASED LABELING FREE APPROACH FOR PROTEOME INVESTIGATIONS

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Surface-activated chemical ionization (SACI) is widely used in the analyses of a wide range of compounds, the main benefits of this technology being its high sensitivity and selectivity as well as its effectiveness under different chromatographic conditions [1, 2, 3]. In this work, firstly, the SACI was coupled with a high-mass-accuracy and high-resolution mass analyzer to characterize the differentially expressed proteins of two *Posidonia oceanica* ecotypes, grown at two different depths under the sea (-5m and -27m). In particular, *P. oceanica* total soluble leaf proteins were firstly separated by one dimensional electrophoresis (1-DE), the protein profile of each ecotype was excised into 24 slices of different molecular weight (MW). All slices were then digested and analyzed by mass spectrometry (MS). Many differentially expressed peptide m/z signals were found (around 200) by comparing MS data from SDS gel slices with the XCMS software and, further, identified in either full scan (MS; peptide mass fingerprint approach) or tandem mass spectrometric (MS/MS; protein identification by similarities of peptide sequences) modes. Secondly, data obtained by SACI approach were compared with those obtained using micro-electrospray ionization (ESI). The SACI strongly resulted to increase the number of detectable proteins. The higher sensitivity of our SACI-based label free approach may be mainly due to the ability of SACI to selectively produce singly charged species of high intensity under 'MS mode' and doubly charged species for 'MS/MS' peptide characterization by simply changing the ionization conditions during data acquisition [4].

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2.2

ANALYSIS OF SOLUBLE OLFACTORY PROTEINS THROUGH AN INTEGRATED MASS SPECTROMETRY APPROACH

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Odorant Binding Proteins (OBP) and Chemosensory Proteins (CSP) are soluble proteins highly expressed in insect chemosensilla where they are involved in perireceptor events leading to Odorant Receptor activation (Pelosi et al., 2006). However, in some species, the expression of such proteins is not limited to olfactory organs, with some being ubiquitous and others being expressed in sex organs or in pheromonal glands, where they are possibly involved in pheromone release. Insect genomes contain a remarkable number of genes encoding OBPs (57 in the malaria vector *Anopheles gambiae*, 44 in the silk moth *Bombyx mori*, 21 in the honeybee *Apis mellifera*), and a lower number of genes encoding CSPs (7) in *An. gambiae*, 10 in *B. mori*, 6 in the honeybee). Until recently, the expression patterns of such genes have been almost exclusively investigated through genomic analysis, while a proteomic approach has been used in only few recent studies (Anholt and Williams, 2009; Dani et al., 2009, 2010).
Using a conventional proteomic strategy, based on micro and nano HPLC- ESI Orbitrap of digested spots from 2D gels, we have analyzed the presence of OBPs and CSPs both in antennae and in pheromonal exocrine glands of different model insects. Moreover, a shotgun proteomic approach, based on nano HPLC-ESI Orbitrap has been used to analyze the antennal extracts of *An. gambiae* for which the conventional technique, using 2D gels, proved difficult to apply due to the tiny size of the antennae.
Finally MALDI-TOF In Source Decay (ISD) TopDown Sequencing (TDS) experiments were performed on some of these proteins, providing useful sequence information

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2.3

QUANTITATIVE PHOSPHOPROTEOME ANALYSIS OF MESODERM-INDUCED MOUSE EMBRYONIC STEM (ES) CELL USING iTRAQ LABELING AND TiO2 PHOSPHOPEPTIDE ENRICHMENT

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Following the initiation of differentiation in culture, mouse ES cells will form embryoid bodies, which generate hematopoietic and endothelial progeny in a temporal pattern, through the presence of a common progenitor with hemangioblast potential. Relative quantification and phosphoproteome analysis represent a great combination to understand the cellular signaling which is suggested to play a major role in the switch between the regulation of pluripotency and differentiation in ES cells. **Methods** Embryonic Stem cell culture and differentiation: the BryGFP+ ES cell line was generated by Fehling et al (1). ES cells were maintained, differentiated and sorted according to the protocols established by the same authors. iTRAQ-LCLC/MSMS analysis: 100μg of nuclear lysates of each sorted cell populations were used for iTRAQ labeling (Applied Biosystem). Phosphoproteins enrichment was obtained using commercial TopTip TiO2 columns. Peptides were fractionated using an SCX (Strong Cation Exchange) column prior to reverse-phase LC-MS/MS using a QStar XL (Applied Biosystem). Data analysis: the data were processed using the Paragon algorithm within ProteinPilot v.3.0 (Applied Biosystem). The phosphoproteome dataset was then integrated and combined with the dataset previously obtained from the total nuclear expression analysis (2). **Results and conclusions** We identified and relatively quantify 455 phosphopeptides but only 23 are significantly changing during ES cells differentiation. Fifteen of these proteins have been also identified in the total nuclear expression analysis. In many cases the phosphorylation reflects the expression, but for several proteins we observed a differential profile compared to undifferentiated cells. We found that no one of the phosphorylated site we identified has already been studied with a functional approach. Some of the proteins in the list are known to be involved in the embryonal development (GSK3, BAF155) but there is still no information about their phosphorylative changes. Work is currently in progress to validate the dataset results using commercially or designed specific phosphoantibodies, Multiple Reaction Monitoring (MRM)-Initiated Detection and Sequencing (MIDAS) and Absolute Quantification (AQUA).

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2.4

WHAT PROTEOMICS CAN DO FOR CULTURAL HERITAGE?

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In the context of artistic and historic objects, the identification of proteins is still a challenging task, because of the very low amount of sample available, because of the complex and quite variable chemical composition of the paints, because of the possible simultaneous presence of several components, and because of degradation of the original materials as a result of aging and pollution. We proposed to adapt proteomic strategies for the identification of proteins in binders of paintings, but also in seeds, food remains in archeological find, etc.., to overcome requirements and difficulties presented by specific samples. In particular, tryptic hydrolysis in heterogeneous phase, followed by the analysis by LC-MS/MS, was successfully used to unambiguously identify milk proteins in a sample from a painting decorating the vaults of the upper church in the Basilica of St. Francis in Assisi (Leo et al., 2009).
Moreover, similar strategies were exploited for the characterization of the natural and unnatural aging products in masterpieces. We identified deamidation as a major modifications of proteins in binders, that could be related to the aging of artistic and historic objects (Leo et al., in preparation).
The systematic analysis of samples from the 14th century frescoes of the Monumental Cemetery in Pisa indicate extensive deamidation occurring on most of the peptides identified, suggesting that the miniature molecular clock, as Robinson and Robinson defined any amide residue present in peptides or proteins (2004), might well be used as molecular marker in artworks.

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2.5

MOLECULAR CHARACTERIZATION OF FOOD-GRADE MEAT PROTEIN HYDROLIZATES

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Animal by-products industry has always been a vital part of the world food production chain, providing valuable new products while reducing pollution loads. Following recent crisis developed in this sector (BSE, dioxin, bird flu) the animal by-product industry had to adapt to newer and stricter regulations. This resulted in an unprecedented availability of animal bioresources. Thanks to the enhancements sought by the European project PROSPARE (PROgress in Saving Proteins And Recovering Energy), the ever growing quantity of unmarketable residues will be transformed in novel products and energy.

The project aims at showing how to obtain and characterise value added peptide mixtures, starting from different raw materials, and how to make them available to industry for further larger scale processing. Such peptide mixtures can be exploited in the food, feed and green chemicals chains. Raw fat materials can be also obtained and transformed in biofuels.

Today many people, principally in the third world countries, is aching for protein deficiency; poultry industry is the most important source of inexpensive meat-derived proteins for human nutrition; in particular meat, bone and feathers residues constitute the lion share of the proteins of the by-products. Mass spectrometry is a valid approach to monitor the composition of the complex peptide mixtures generated by developing technologies.

Characterization of sequences of most abundant peptides in the mixture had the aim to check the safety of developed protein-rich supplement before it will be market and control a new generation of food ingredients with designed properties addressed to different industrial sectors.

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2.6

SOLID PHASE ISOBARIC MASS TAG REAGENT FOR SIMULTANEOUS PROTEIN IDENTIFICATION AND ASSAY

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The development of reliable methodologies for simultaneous identification and quantitation of a large numbers of proteins is an important challenge in proteomics field. Stable-isotope labeling methods is the most broadly applied for sample preparation due to their high accuracy for relative quantitation [1]. Stable isotopes are generally introduced into proteins or peptides by metabolic labeling strategy (SILAC), [2] enzymatic ^{18}O-labeling of peptide C-termini, [3] chemical modification of specific functional groups using reagents such as isotope-coded affinity tags (ICATs).[4] Amine- reactive isobaric tags (TMSs) [5] and isobaric mass tagging reagents (iTRAQ) [6] have been designed to overcome these limitation.

In this report, two-plex solid-phase isobaric label-free reagents based on “pseudo” tripeptides are proposed for quantitative protein analysis. These reagents attach isobaric mass tags at the N-termini of digest mixtures peptides to perform relative quantification. The solid-phase isobaric mass tagging (SPIMT) reagents were designed according to the principle that they must be isobaric and label-free rather than labeled with expensive heavy atoms. Consequently, all derivatized peptides are isobaric and yield signature or reporter ions after CID, that can be used to identify and quantify individual members of the two-plex set. Free amino acids, glutathione, and peptides mixture obtained using different protease on BSA, Transferrin and Lactotransferrin were checked to study the chemical reactivity of the SPIMT reagents, the linearity of quantification and the effect of the amide tripeptide tags on the fragmentation pattern. The methodology was extended to a mixture of standard proteins prepared at different concentration level.

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2.7

PECULIAR FEATURES OF STABLE ISOTOPE LABELING VIA DIALKYLATION OF PEPTIDES FOR RELATIVE QUANTITATIVE ANALYSIS IN LC-MS EXPERIMENTS

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A relative quantification of the expression of proteins in biological samples is one of the most crucial issues in proteomics. Several approaches have been developed in the last decade, focusing on stable-isotope labeling of peptides and protein, in order to carry out quantitative analysis in LC-MS or LC-MS/MS experiments. The reaction of peptides with formaldehyde or formaldehyde-d_2 and $NaCNBH_3$ has been reported to be a clean reaction, highly specific for terminal NH_2 and lysine residues, and relatively not expensive if compared with the commercially available kits for isotope coding. Furthermore, isotopic effects in LC are negligible and a separation of at least 4 m/z for singly charged ions allows to easily individuate couples of peaks in the mass spectra of mixtures containing light and heavy labeled samples.[1] Acetaldehyde and acetaldehyde-d_4 can also be employed to obtain a wider separation between mass peaks, namely adding four ethyl groups instead of methyl ones.[2] We report on a series of observations showing how tertiary amino groups generated in the dimethylation or the diethylation reactions can undergo undesired rearrangements leading to an incorrect measurement of the relative amount of a peptide in quantitative analysis. Beside a possible immonium ion formation, due to a matrix effect in the Laser Desorption Ionization,[3] the presence of a $[M - H]^+$ signal has been observed in ESI-MS experiments as well. With a systematic investigation, based on changing reaction conditions, MALDI matrix and peptide features, we wish to rationalize this scenario.

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2.8

EXPLORATION OF THE "HIDDEN" PROTEOME OF DONKEY'S MILK VIA COMBINATORIAL LIGAND LIBRARIES TECHNOLOGY

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Recent clinical studies have clearly demonstrated that the use of donkey's milk (DM) in the diet of children allergic to cow's milk proteins represents, for a large number (83-88%) of the investigated subjects, a unique safe and resolving "natural" treatment [1-3]. Consequently, DM has recently aroused scientific and clinical interest, above all among pediatric allergologists. Notwithstanding this renewed growing interest, the characterization at molecular level of DM is still scant. The wider information concerns the major components of whey proteins, whereas the investigation of caseins is still at a relatively early stage of progress [4]. On the contrary, nothing is known about the minor or trace protein components of DM. As a consequence, until today the molecular basis for the demonstrated absence of allergy of this milk for a large number of cow's milk allergic subjects, as well as the residual allergy for a minor number of individuals, remains completely unknown. Classical approaches such as pre-fractionation and immuno-depletion methodologies are frequently used to remove the most abundant proteins. Unfortunately, these methods are unable to concentrate trace components, which could remain below the detection limits of analytical approaches, and may even cause non-specific depletion of low-abundance components. We report here the application of the novel combinatorial peptide ligand libraries technology [5-7], aimed to the investigation, discovery and identification of previously unknown protein components belonging to the "hidden" proteome of donkey's milk.

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2.9

APPLICATION OF MSB-MALDI-TOF-MS TECHNOLOGY TO BODY FLUIDS AND TISSUES PEPTIDOME PROFILING

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The design and generation of material-based platforms for capturing "molecular signatures" from tissue and body fluids have gained increasing interests in recent years.
For a MS-peptide profiling method to be successful, two criteria need to be fulfilled: the method of sample preparation needs to be selective, removing salts and large biomolecules interfering with the analysis, which must be very sensitive.
Recently, we have described an approach based on mesoporous silica beads (MSB) for selective binding and enrichment of plasma low molecular weight proteins that allows salts and high abundant proteins depletion. Such platform is based on combined utilization of MSB and MALDI-TOF-MS. This platform has been extended with direct functionalizaton of MSB with different chemical groups in order to interact with specific subset of peptides and low molecular components present in plasma and urine peptidome.
We are now extending our platform to generate comprehensive peptide profiles from synovial fluid, characterized by high content of hyaluronic acid.
Another research line is devoted to the study of induced sputum, in which the presence of salts and abundant, high-molecular-weight mucins complicates its processing.
Finally preliminary investigations on the cerebral tissue of hibernating *Mesocricetus auratus* are directed to test the ability of MSB to profile still unknown peptidome, thus elucidating neuronal mechanisms implicated on the induction of hibernation.
The use of MSB, while not capturing all possible peptides and metabolites, nevertheless appears to reveal complex and rich fingerprints which represent a direct avenue to examine the (patho)physiology of many biological fluids and tissues.

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2.10

PHENOTYPIC PROFILE LINKED TO ZN INFLUX SYSTEM INHIBITION IN *SALMONELLA ENTERICA*: PROTEOMICS INVESTIGATIONS AND METAL PROFILING

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Zinc is required for a wide variety of cellular functions and plays a key role in bacterial metabolism and virulence. However Zn can also be toxic and its influx is tightly regulated in bacteria. The high affinity zinc uptake transporter ZnuABC is the main Zn influx control system in *Salmonella enterica* under conditions of Zn starvation. It has been shown that deletion of the gene encoding for its periplasmic subunit ZnuA significantly affects *S. enterica* serovar Typhimurium growth rate and virulence[1] highlighting the importance of this system in the pathogen-host interaction. To gain further insight into the mechanisms involved in Zn influx regulation and better understand the role of the ZnuABC system in *S. enterica*, we proposed a label free shotgun proteomics analysis to compare the protein expression levels in *S. enterica* wild type and *znuA* knockout growing both in standard conditions and under Zn starvation. Functional analysis revealed that the *znuA* mutant strain overexpressed many proteins related to metabolism and to the management of genetic information, whereas proteins related to the environmental information processing were found to be significantly down-regulated in both growth conditions. Furthermore, several of the differentially regulated proteins are predicted metal-binding proteins. Therefore, we tentatively assessed the functional impact of this differential protein expression on the element profiles of the same *S. enterica* strains by inductively coupled mass spectrometry (ICP-MS). This open platform investigation found significant alterations in the *znuA* mutant strain that are reversed by Zn supplementation. In conclusion, our data demonstrate that inhibition of Zn influx has relevant effects either on the bacterial proteome or on the element profile and shed new light on the role of the ZnuABC system and Zn influx in *S. enterica* pathogenicity.

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2.11

3DSPECTRA: A 3D QUANTIFICATION SOFTWARE FOR LC-MS LABELED PROFILE DATA

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Mass spectrometry-based proteomics generates high informative but challenging to parse datasets, such as profile 3D-LC-MS data: LC-MS separates peptides in two dimensions (t, m/z) minimizing their overlap, and the profile acquisition mode enhances signal quantification. To exploit both data features, we developed 3DSpectra, a Matlab quantification software for LC-MS labeled profile data.
3DSpectra accesses data using mzRTree [1], a data structure optimized to efficiently manage profile data, pivotal for quantification. 3DSpectra makes use of a priori information provided by search engines to quantify identified peptides, whose metadata are stored in a structured collection. Retrieved the peptide data, 3DSpectra recognizes peptide peaks borders, via a 3D Finite Mixture Model based on the isotopic distribution, and processes and quantifies the clustered data-points. After outlier removal, performed either automatically or manually, the obtained results, their statistics and regression lines, between light and heavy volumes, are saved to Excel and .ps files.
3DSpectra quantification performance has been assessed employing a controlled mixture of ICPL-labeled proteins, acquired in enhanced profile mode, mixed at different ratios in triplicates. 3DSpectra regression analysis showed high linearity, 0.1% significance, and slightly better regression coefficients than ASAPRatio (MASPECTRAS [2] improved implementation) on the same set of peptides. Above all, 3DSpectra quantifies from 2 to 4 times more peptides on the differentially expressed ratios, yielding a 100% to 300% gain in quantification efficiency, defined as the ratio of quantified peptides to identified peptides. Therefore, 3DSpectra provides a reliable quantification strategy and wider proteome coverage at the level of peptide quantification.

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2.12

iTRAQ CHEMISTRY TO INVESTIGATE PROTEIN CARBONYLATION

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Carbonylation is a non enzymatic irreversibile post-translational modification of proteins due to the presence of excess of ROS (Reactive Oxygen Species) in cells [1-2]. Carbonylation of specific amminoacidic side chains is one of the most abundant consequences of oxidative stress; so determination of the content of carbonyl groups in proteins is regarded as a reliable way to estimate the cellular damage caused by oxidative stress. For this reason several methods have been developed to determine and quantitate protein content of carbonyl groups [3-4]. We present in this work a novel methodology for selective labeling of carbonyl groups in proteins and selective analysis by advanced mass spectrometric techniques; in particular we use dansylhydrazide and iTRAQhydrazide for labelling of carbonyls, a dansyl and an iTRAQ derivatives specifically reactive towards carbonyl groups. This method was proved to lead to the simultaneous localisation and quantification of modified sites both in model proteins and in biological systems. Here the strategy proposed, taking in account the availability of linear ion trap to select specific labeled peptides giving rise to diagnostic MS/MS product ions, resulted to be of interest in the selective detection of protein carbonylation. Moreover, because of its operational simplicity, avoiding long-lasting and time-consuming fractionation procedures, this new strategy seems to be well suited for large-scale quantitative profiling of PTMs.

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2.13

DEVELOPMENT OF A MULTI-STEP ENRICHMENT PROTOCOL FOR ISOLATING SUBPROTEOMES FOR DIAGNOSTIC/PROGNOSTIC PURPOSES

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Mass-Spectrometry-based clinical proteomics is a promising evolving field, though its efficacy for disease biomarker discovery is mainly hampered by the wide dynamic range in protein concentration in body fluids. Although the performance of analytical instruments is rapidly improving, many efforts are still required to mine all necessary information in a single chromatographic run in the so-called "shotgun proteomics". Thus, several preparative strategies are emerging to prefractionate complex biological specimens into subproteomes [1], more suitable for HPLC separation and mass-spectrometry analysis.

Here we present a two-step prefractionation procedure of the human serum proteome to harvest low abundant peptides. First, sera were immunodepleted from the twelve most abundant proteins [2] and the resulting concentrated eluates were then treated with beads coated with a hexapeptide combinatorial ligand library [3]. Moreover, we tuned up a detergent-free protocol to recover the digested enriched peptide fraction from coated beads, by directly processing the samples through the canonical preparative steps for bottom-up MS proteomics, thus avoiding any further purification and concentration step before MS analysis.

In our hands the described approach provided better results than applying the coated beads straight to non-depleted serum; indeed, we found that coated beads react with several most abundant proteins somewhat compromising their enrichment feature.

The presented multi-step enrichment protocol was benchmarked by analysing serum fractions from healthy subjects and patients suffering from cirrhosis and/or hepatocellular carcinoma; this tumour represents a major treat for public health, being a high-ranked cause of death for cancer.

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3.1

SUBCELLULAR PROTEOMIC ANALISYS OF ENRICHED ER AND GOLGI FRACTIONS DURING TOMATO FRUIT RIPENING

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The Golgi complex is the central organelle of the secretory pathway and functions to posttranslationally modify newly synthesized proteins and sort them, packaged in vesicles for transport to their sites of function. Our aim is monitoring the pattern of protein expression along the secretory pathway during tomato fruit ripening, and identifying proteins of the endoplasmic reticulum (ER) and Golgi apparatus that are differentially expressed. ER and Golgi are crucial for the secretory pathway and play a central role in fruit ripening; in addition the Golgi is responsible for the production of the non cellulosic component of cell wall. ER and Golgi microsomal vesicles were separated by centrifugation through different types of density gradients. Assuming that an organelle has a unique distribution pattern it is possible to identify ER or Golgi components by comparing their enrichment using known markers of these compartments. Profiling of protein distribution in different stages of ripening of tomato fruits was determined by DIGE analysis of gradient fractions, respectively enriched in the ER and Golgi microsomes, to allow quantitative evaluation of differentially expressed proteins. Moreover, to overcome technical limitations resulting in reduced identifications of transmembrane proteins and posttranslational modifications, we have developed an alternative multidimensional identification strategy using a combination of one dimensional electrophoresis and nanoLC-ESI-MS/MS.

3.2

CHARACTERIZATION OF *PLASMOPARA*-SUSCEPTIBILITY IN GRAPEVINE BY PROTEOMIC ANALYSIS

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We have carried out a proteomic 2D analysis of *Vitis vinifera* leaves cv. Pinot Noir artificially infected with *Plasmopara viticola* (30000 sporangia per ml), and collected at 24, 48 and 96 hours post-inoculation. Leaf tissues from 3 independent biological replicates were pooled. After testing different protocols for protein extraction, good quality 2D maps were obtained by performing protein extraction in a solubilizing/lysing solution containing 7M urea, 2M thiourea, 80mM citric acid, 1% C7Bz0 and 1x protease inhibitor cocktail. Gels (five per each sample) were scanned using a Bio-Rad VersaDoc 1000 imaging system and analyzed by PDQuest software (Bio-Rad) for spot detection, background subtraction and spot OD intensity quantification. After normalization and statistical analysis, about 100 spots differentially expressed were identified (81 up-regulated and 19 down-regulated, fold-change ≥ 2, with $p \leq 0.05$). Identification by RP-HPLC-ESI-MS/MS analysis, revealed 72 unique proteins. Some of these modulations were validated by RT-PCR. Functional categories prevalently affected by infection were related to Calvin cycle and glycolysis (28%), oxidative stress response (19%), defence response (12%), photosynthesis (9%) and protein folding (6%). A peculiar pattern in the number of modulated protein over time was observed: 54 and 51 differentially expressed proteins were detected at 24 hpi and 96 hpi, respectively, and only 16 at 48 hpi, none of which included in the “defence response” category suggesting a temporary break down of the response, that will be the object of further investigations.

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3.3

GLYCOPROTEOME CHARACTERISATION BY INTEGRATED MASS SPECTROMETRY TECHNIQUES

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This work aims at the development and setting up of new methodological platforms based on the integration of affinity chromatography methodologies and multistage mass spectrometry (MS^n) for the study of glycoproteome.
We set up a method based on the reaction of carbohydrates with hydrazine derivatives leads up to hydrazone derivatives. Derivatization is achieved by the use of dansylhydrazine which introduces a strong basic group improving ionization. Furthermore precursor ion scan analyses can exploit dansyl-derivatives ability to generate specific product ions at m/z 170 and 234 in MS^2 and a characteristic fragmentation pattern at m/z 234→170 in MS3. The procedure has been set up on synthetic oligosaccharides, revealing that dansyl derivatization improves fragmentation properties in MS. The procedure was applied to oligosaccharides extracted by white and yolk egg glycoproteins. We have shown that oligosaccharides coming from yolk are structurally related to the ones from the white, even sialylated.
Glycopeptide component was enriched by ConA affinity purification after tryptic digestion to simplify the mixture for N-glycosylation site selection and identification. The glycopeptides were then deglycosylated by PNGase F and analysed by LC-MS/MS. The majority of identified peptides contained the conserved N-linked glycosylation motif, indicating that N-glycosylation peptides were isolated with high selectivity. The presence of a putative N-glycosylation site was confirmed by the fact that peptides mass was increase of 1 Da, due to the conversion of Asn into Asp after PNGase F incubation. This procedure was applied to *Arabidopsis thaliana* giving new highlights in the elucidation of vegetal glycoproteome.

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3.4

ALLERGENS OF THE SALT-SOLUBLE FRACTION OF THE GRAIN ARE NOT MODIFIED IN A GM LINE OVER-EXPRESSING A GLUTENIN LOW MOLECULAR WEIGHT SUBUNIT

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Wheat is one of the 6 groups of food identified by the Alimentarius Codex as being responsible for 90% of food allergies. Food allergy is an adverse response to food triggered by the body immune system. The allergens responsible for wheat allergy are proteins accounting for about 10-15% of the grain dry weight [1, 2, 3]. Therefore, much attention is now being focused on foods from genetically modified (GM) plants because of the risk of allergenicity. In Europe, there is considerable public resistance to the use of GM technology for crop improvement. This resistance includes the perception that the insertion of transgenes into host plant genomes may result in unpredicted effects on the expression of other genes and effects on plant phenotype (e.g. increases in toxins and allergies). If this is true, transgenic crops could not be considered "substantially equivalent" to non-GM crops [4]. The objective of this preliminary work is to investigate if the event of transgenesis has an effect on allergenic potential.
For this study sera from children and adults with clinically documented wheat allergy are used for a comparison between a GM-wheat and its wild-type counterpart along with another cultivated wheat. The investigation is focused on the soluble protein fraction of wheat. For the comparison, ELISA test and 2D immunoblots are used.
Data show that there is no significant difference in the amount of allergenic polypeptides present in the salt-soluble fraction between the GM genotype and its non-transformed counterpart, as well as in the commercial genotype here considered.

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3.5

LEAF PROTEOME OF MARINE PLANT, *POSIDONIA OCEANICA,* REVEALS SOME INSIGHT ON ACCLIMATION TO DEPTH

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Posidonia oceanica is a marine plant which can live up to -40 m in clear waters. This poses the question about how *P.oceanica* could adapt to such uncommon environment for higher plants. The Expressed Sequence Tags database of *P. oceanica* is now available[1], enabling a proteomic approach to better understand the physiology of this plant. We successfully applied the highly innovative USIS[2] mass spectrometry coupled with off line 1D-SDS electrophoresis[3] and labeling free approach to *P.oceanica* growing at -5 m and -27 m depths[4]. Among the 90 identified proteins, some are highly expressed in the shallow plants, have not been found in deep plants. It is of interest the phosphatidylinositol 3-kinase that is involved in phosphoinositide-mediated signaling in response to salt stress. Plants acclimated to depth over-express 9 proteins including the ubiquitin-carboxylate terminal hydrolase 12, involved in protein fate by the ubiquitin/proteasome pathway. The differential expression of proteins involved in organelle function and turnover (Pentatricopeptide repeat-containing protein At3g098060, NAD(P)H-quinone oxidoreductase subunit, Glutamyl-tRNA reductase, Nudix hydrolase) may be related to (i) a higher photosynthetic demand for a more efficient transformation of sunlight into chemical energy, (ii) a control of the levels of metabolic intermediates and/or signalling compounds, and (iii) a transcriptional regulation of some genes related to chlorophyll and porphyrins biosynthesis. Finally, an over-expression of cytosolic Phosphoglycerate kinase and chloroplastic Glyceraldehyde-3-phosphate dehydrogenase strongly suggests the increase in the rate of photosynthetic electron transport and CO_2 fixation.

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3.6

EFFECT OF DROUGHT STRESS ON THE EXPRESSION OF METABOLIC PROTEINS IN DEVELOPING GRAINS OF DURUM WHEAT

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Durum wheat (*Triticum durum* Desf.) is one of most relevant crop in the Mediterranean basin and represents the basis of several traditional foods. Mediterranean climate, characterized by sudden increases of temperatures and water deficit in spring time, during grain filling, may limit the optimal development of this crop with repercussions on grain yield. Moreover, heat and drought stress occurring during the grain-filling, can alter seed protein accumulation influencing both crop yield and yield quality.

Albumins, globulins and prolamins represent the principal protein groups in wheat endosperm. The non-prolamin fraction includes proteins with metabolic activity often implicated in allergies or intolerances in sensitive consumers. Prolamins (gladins and glutenins) constitute the gluten and their pattern influences rheological properties of dough.

This work aims to investigate the effect of drought stress on metabolic protein expression in durum wheat. Two commonly cultivated Italian cultivars (Svevo and Ciccio) were subjected to two irrigation regimes (well watered and water stressed) in growth chamber. Grain samples were collected at three grain-filling stages (Milk, Dough and Ripening stages [1]) and seed metabolic protein expression was analyzed through 2D electrophoresis; spots were revealed with Coomassie Brilliant Blue and then processed with Progenesis SameSpots software.

Preliminary analyses performed on the Milk stage show that most of the differential polypeptides after drought stress are in common with heat stress [2], as expected. Dough and Ripening stages are under investigation.

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3.7

AN INSIGHT INTO EMMER (TRITICUM DICOCCON SCHRANK) MATURE SEED SOLUBLE PROTEOME

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The Italian word "farro" indicates a group of cereals whose seeds are characterized by a hulled habit. After threshing, their grains still appear covered by the spikelet glumes. Differences in their ploidy levels are quite distinctive in that these species are diploid, tetraploid and hexaploid, and correspond, respectively, to the hulled wheats commonly known as einkorn, emmer and spelt. Emmer is the farro species commonly cultivated in Italy [1].

The cereal seed, besides the embryo, comprises the endosperm tissue which in turn is constituted by the bulky starchy endosperm (flour) and the surrounding aleurone (bran). The aleurone tissue contains exclusively soluble storage proteins that, on the other hand, represents only a minor fraction of the starchy endosperm total proteins. In mature seeds the starchy endosperm is made of dead cells while the aleurone cells mantains full vitality and plays many regulatory functions in the seed physiology; these functions are mediated by several soluble proteins. Among these proteins are also included factors possibly responsible for the emmer adaptability to adverse ecological conditions that, on the other hand, may result in compounds negatively affecting human nutrition (antinutrients and allergens). We analyzed and compared aleurone and starchy endosperm soluble proteins 2D-maps, through in-gel digestion, mass spectrometry (MALDI-TOF MS and RP-HPLC/n-ESI-MSMS) and bioinformatics screening, in order to reveal possible patterns of qualitative differential expression.

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3.8

STARCH-BOUND 2S PROTEINS IN *AVENA SATIVA*

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The 2S superfamily seed proteins consist in diverse proteins which maintain a characteristic framework of ten cysteine residues involved in intramolecular disulfide bonds. This family includes Ns-LTPs, indolines and amilase/trypsin inhibitor. Common wheat seed indolines are composed of two main isoforms, approximately 13KDa in size, puroindoline-A (PIN-A) and puroindoline-B (PIN-B) encoded by *Pina-D1* and *Pinb-D1* genes, respectively, occurring at the *Hardness* (*Ha*) locus on the short arm of chromosome 5D. Their name was given in regard to the unique tryptophan-rich domain (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys) of the Pin-A. This domain is truncated in PIN-B (Trp-Pro-Thr-Trp-Trp-Lys). Puroindolines genes were found in oats, barley and rye. Variations in puroindoline composition affect grain hardness, crumb structure and rheological properties of wheat dough[1].

Oats *(Avena sativa)* exhibits an extremely soft endosperm. Previous data suggest that oats contains a group of starch-granule-bound proteins, structurally similar to puroindolines and named vromoindolines (Vins). In A-PAGE X SDS-PAGE, Vins appeared as two groups of polipeptides, A and B, (13 KDa), each group containing three major component. Vins B proteins contain 10 cysteine, have a basic pI and show a tryptophan-rich domain of four tryptophan residues. 2D-spots relative to Vin A and Vin B group were excised and submitted to tryptic proteolysis; peptide mixtures were then analyzed by MALDI-TOF MS and RP-HPLC/n-ESI-MSMS. Peptide sequences were deduced by interpretation of MSMS data. The deduced sequences were used to construct a mix of primers in order to reveal gene coding by 5' and 3' RACE (Rapid Amplification of cDNA Ends) on a cDNA seeds library.

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3.9

STUDY OF THE MATURATION PROCESS OF LOW MOLECULAR WEIGHT GLUTENIN SUBUNITS BY MEANS OF TRANSGENIC WHEAT PLANTS AND PROTEOMIC APPROACHES

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The glutenin polymers of wheat endosperm are made up of two main types, the high- (HMW-GS) and low- (LMW-GS) molecular weight subunits; subtypes of LMW-GS include LMW-m, LMW-s, and LMW-i, named according to the first amino acid of the mature sequence [1].
The main difference between LMW-m and LMW-s is the absence in the mature LMW-s type of the expected first 3 N-terminal amino acids (MET-) characteristic of the LMW-m type. According to algorithms that predict the signal cleavage site, the presence of N instead of T in LMW-s was the basis for our hypothesis that a differential processing of the N-terminal end of the LMW-s sequence occurs such that cleavage at the N residue by an asparaginyl peptidase generates the observed N-terminus of the LMW-s type, similar to the processing that apparently occurs in ω-gliadins [3].
In order to investigate this possibility, we produced transgenic wheat lines, transformed with mutated versions of the LMW-m and LMW-s genes, such that N was substituted for T in the LMW-m gene and T for N in the LMW-s gene, for comparison with their wild type counterparts. Western Blot analyses, combined with proteomic comparisons (by MS-MS) between the transgenic and untransformed lines, have enabled us to determine that the processing occurred according to our predictions for the LMW-m construct with cleavage occurring at the introduced N. Further analyses of the mutated version of the LMW-s type and the wild type polypeptides are in progress.

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3.10

IDENTIFICATION OF WHEAT CHROMOSOME 5A ENCODED POLYPEPTIDES BY A PROTEOMIC APPROACH

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In the frame of the Italian project "Physical map of wheat chromosome 5A: Italian initiative for the sequencing of the whole genome", that is part of the international initiatives ETGI & IWSC, our activity regards the analysis of the chromosome 5A proteome.
Genes present on the chromosome 5A so far identified have been shown to influence kernel protein content, frost resistance and hardness, but the great majority of them is still unknown.
The wheat kernel proteome consists mainly of gluten proteins that are encoded by genes present on the homoeologous group 1 and 6 chromosomes, and by soluble proteins, including polypeptides with structural and metabolic functions, whose chromosome location is mostly unknown. Among these latter, also chloroform-methanol (CM) polypeptides are included.
We have started this project with the analysis of 5A encoded polypeptides present in tetraploid wheats: the disomic substitution line of chromosome 5A of *Triticum dicoccoides* (with genome AABB, as for durum wheat) in the durum wheat cv. Langdon (LDN(DIC-5A)) has been used.
Both metabolic and CM proteins [1] were submitted to 2D proteomic mapping allowing to identify the polypeptides associated to chromosome 5A. The comparison has in fact revealed 21 spots for the metabolic fraction and 17 spots for the CM-fraction that are encoded by genes present on chromosome 5A of *T. dicoccoides*, and 3 spots for the metabolic fraction and 6 spots for the CM-fraction (encoded by genes at 5A chromosome) in *Triticum durum* cv. Langdon.
MS characterization of the identified 5A encoded polypeptides is under investigation.

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3.11

A PROTEOMIC APPROACH TO ASSIGN DISULPHIDE BONDS OF THE HIGH MOLECULAR WEIGHT GLUTENIN SUBUNIT Bx7

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The technological properties of wheat flours are related to the composition of kernel storage proteins [1]. The association of prolamins (gliadins and glutenins) in gluten complex is related to disulphide and non covalent bonds formation. In particular, the monomeric gliadins, with intra-molecular or no disulphide bonds, interact via non-covalent interactions and contribute to viscosity of doughs; glutenins, instead, are organized in large polymers by means of intra- and inter-molecular disulphide bonds and are responsible for strength and elasticity of flours. The importance of disulphide bonds in maintaining the structure/functional properties was demonstrated in experiments by the addition of reducing agents to glutens, resulting in weaker doughs and in the increased solubility of glutenins [2]. Unfortunately, the knowledge of the position of intra- and inter-molecular disulphide bonds is not complete, and the relationship between structure and function is poorly understood. In preliminary studies the structure of glutenins aggregates was examined analyzing their depolymerisation behaviour by stepwise chemical reduction. It was observed that during reduction there is a non-random release of subunits and the presence of particular oligomers suggested that glutenin polymers have a well-ordered structure [3,4].
In this work we report the assignment of disulphide bonds in dimers of Bx7 HMW-glutenin subunits, using a line of durum wheat cv *Svevo* possessing only the Bx7 HMW glutenin. A procedure based on 2D gels (sample partially reduced *vs*. fully reduced), differential alkylation, enzymatic hydrolysis, followed by MALDI TOF-TOF analysis has allowed to assign both intra- and inter-molecular disulphide bonds in this glutenin subunit.

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3.12

COMPARISON OF PROTEIN VARIATIONS IN *THLASPI CAERULESCENS* POPULATIONS FROM METALLIFEROUS AND NON-METALLIFEROUS SOILS

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In this work we analysed the protein variations which occurred in two *Thlaspi caerulescens* populations when subjected to 0 and 10 μM nickel (Ni) treatments: the Ni hyperaccumulator *T. caerulescens* from a metalliferous soil in Italy and *T. caerulescens* from Czech Republic, adapted to grow on a non-metalliferous soil. Ni accumulation in roots and shoots and the effect on growth and morphology were examined. Leaves proteins profiles of Ni treated and untreated samples were analysed by two dimensional liquid chromatography technique. From the comparison of more than 500 proteins, few differences were observed between treated and untreated plants of the same population. Differences were found between the two *Thlaspi* populations, instead. Proteins involved in transport, metal chelation and signal transduction increased in abundance in the 10 μM Ni treated samples while, in condition of absence of Ni, proteins involved in sulphur metabolism, protection against reactive oxygen species and stress response showed to increase in abundance in the two populations. These proteins can be used as biomarkers both for monitoring biodiversity in indigenous plants and for selection of Ni phytoremediation plants.

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3.13

ANALYSIS OF REDOX-RELATED PROTEINS IN BARLEY SEED PROTEOMES

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Protein profiles of barley seed tissues are analysed during grain filling, maturation, germination and radicle elongation, and several hundred barley seed proteins have been identified [1]. Thioredoxins are protein disulphide reductases that regulate the intracellular redox environment and processes including DNA synthesis, oxidative stress responses and apoptosis. The ascorbate-glutathione cycle plays a central role in maintainance of cellular redox balance and is likely to include interactions with the thioredoxin system. Functional proteomics approaches focusing on the seed ascorbate-glutathione cycle and thioredoxin system will be described. Identification of potential thioredoxin target proteins and structural studies provide insight into determinants for target protein recognition by thioredoxin [2].

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3.14

PROTEOME ANALYSIS OF LENTIL (*LENS CULINARIS* MEDIK.) SEEDS UNDER SALT STRESS

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Lentil is an important crop and a source of protein for human nutrition. Over time, local constraints have produced a myriad of different landraces within the *Lens culinaris* species. However, in the modern countries, the intense economical transformation focussed the agriculture on more remunerative crops, inducing a progressive reduction of cultivation of lentils and the disappearance of several local populations. In a previous paper the proteome map of lentil mature seed was deciphered and several proteins have been proposed to be "landrace markers" since they discriminate local landraces (Capracotta and Conca Casale) from widely spread commercial varieties [1]. To further characterize the local landraces we evaluated their response to abiotic stress at phisiological and biochemical level. In particular we have investigated the effect of the NaCl on seed germination and used a proteomic approach to identify proteins that may play a role in the adaptation to saline conditions. Results show that the salt treatment affects the germination rate and percentage of all the lentil populations analysed. The quantitative analysis of the 2-DE gels of control and stressed seeds revealed 126 differentially expressed protein spots. The comparison with the deciphered proteome map of lentil mature seed allowed the identification of 52 proteins differentially expressed involved in the storage and disease function.

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3.15

THE ASCOMYCETE *CERATOCYSTIS PLATANI* IS THE CAUSAL AGENT OF THE CANKER STAIN DISEASE. THE SET UP OF A PROTEOMIC STUDY.

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Cerato-platanin (CP) is a moderately hydrophobic protein abundantly secreted and localized in the cell wall of *C. platani;* it is believed to play a role in host–plant interaction, inducing cell necrosis and/or defense responses such as phytoalexin synthesis, overexpression of defense-related genes, induction of H_2O_2 and NO and restriction of conidia growth[1,2,3]. Therefore, CP acts as PAMPs (Pathogen Associated Molecular Patterns) activating a primary defence system that is highly effective against the most potential pathogens[4]. Moreover, CP is the founder of the "cerato-platanin family" whose members are secreted proteins (75-120AA) involved in the microbe-host interaction acting as phytotoxins, elicitors of defense responses and allergens[5,6].
The proteomic project that we are setting up has the aim to investigate the role of CP both in the physiology of the fungus and in the pathology of the canker stain disease.
Conidia of *C. platani* were harvested from fruiting cultures and suspended in PDB (about $2x10^5$ conidia ml^{-1}). 100 ul droplets were then applied to a plastic sheet and to plane leaves obtained by in vitro micro-propagation. All samples were maintained in a moisture chamber at RT. After 48h the mycelium was removed and lyophilized and leaves were put at -80°C for further analysis. 0.05 g of dry mycelium was re-suspended in an acidic extraction buffer containing dodecyl-maltoside as a detergent. Results from 2D-gels will highlight proteins expressed in the fungus grown in PDB *vs* fungus grown on planta in order to investigated the role of CP as a PAMP.

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3.16

THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS AFFECTS PROTEIN EXPRESSION IN THE LEAVES OF AN ELITE POPLAR CLONE GROWN ON HEAVY METAL POLLUTED SOIL.

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Copper and zinc are essential nutrients, but at high concentration they become toxic to plants, inhibiting various physiological and biochemical processes. The ability of some poplar genotypes to tolerate soil pollutants, and especially heavy metals, can be improved by the symbiosis with arbuscular mycorrhizal (AM) fungi[(1)], which are naturally present in the roots of most plant species.
In this work, a previously selected, heavy metal tolerant, clone of *Populus alba* L.[(2)] was used to investigate the effects of the symbiosis with the AM fungus *Glomus intraradices* on the leaf protein expression. Plants were maintained in a glasshouse for two growing seasons, either in soil from an industrial area (copper and zinc-contaminated) or in unpolluted soil. Leaf samples were collected at four, six and sixteen months of growth. To perform proteome analysis, poplar leaves were ground in liquid nitrogen and proteins were extracted with TCA/acetone precipitation[(3)]. All samples were separated by 2-DE using linear pH 3-10 and 4-7 IPG strips. Comparative analysis was performed with Same Spot software and results were statistically analysed by ANOVA. Identifications were carried out by nano-LC ESI Q-TOF MS/MS, followed by searching the NCBInr database.
About 400 proteins were reproducibly separated from the samples at each harvest. At the first harvest the most relevant effect on protein modulation was exerted by the AMF, at the second one by the metals, and at the last one by both treatments. The modulated proteins are mostly involved in photosynthesis, sugar and glutathione metabolism, oxidative damage and protein synthesis.

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3.17

CHARACTERIZATION OF ANTENNA ISOFORMS FROM SPINACH LEAVES GROWN UNDER DIFFERENT LIGHT CONDITIONS.

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The major light-harvesting complex (LHC-II) of higher plants plays a crucial role in capturing light energy for photosynthesis and in regulating the flow of energy within the photosynthetic apparatus. Native LHC-II isolated from plant tissue consists of three isoforms, Lhcb1, Lhcb2, and Lhcb3, which form homo- and heterotrimers. Usually plants are exposed to environment where light and temperature conditions are largely variable. Among all the environmental parameter light intensity has a major influence on plants life.When the plants are exposed to high excess of light to optimize performance and to avoid photodamage releases a monomer of LHCII from PSII to PSI preferentially in phosphorilated form [1]. During light stress at 100 $\mu E\ m^{-2}\ s^{-1}$ plants must balance the excitation of the two photosystem and so there is a migration of light-harvesting complex type 2 (Lhcb2) from grana to PSI. Changes in the association-dissociation state of LHCII are the regulatory mechanism that optimize light-harvesting function under different stress condition. In this communication we want to investigate the behaviour of antenna proteins (Lhcb) during different acclimatation, then to correlate the expression of the Lhcb1 isoforms to relative mRNA. For this aim we used a microRotofor (Biorad) that avoid detergent and we obtained the antenna proteins in their native form. Separation of complexes is based on the protein's real physicochemical properties After wards a reverse-phase HPLC method on line with mass spectrometry allowed us to have a qualitative and quantitative analysis of Lhcb proteins.

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3.18

2D-LC AS A SUITABLE TECHNIQUE FOR COMPARATIVE PROTEOMICS OF DIFFERENT PLANT SPECIES IN RESPONSE TO METAL CONTAMINANTS

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In many plant species subjected to metal stress a network of proteins and biochemical cascades that may lead to a controlled homeostasis of metals has been recently identified. There is no protein technology that can be as sensitive as transcripts microarray analyses, thus it is necessary to combine different protein separation techniques to enrich proteomic data. In this work we tested a 2D-LC system for its ability to identify proteins regulated by excess of metals in different plant species. The use of liquid protein mapping instead of gel mapping, to scan many samples for important or landmark proteins has some advantages: *i)* the possibility to load protein crude extracts, avoiding purification steps and limiting the risk to loose less abundant proteins in the sample; *ii)* the higher amount of proteins that is loaded in a single run (up to 10 mg of protein extract), could reveal also less abundant proteins with regulatory functions; *iii)* working with liquid protein fractions is more suitable for a direct identification by MS techniques; *iv)* the automation of the system allows for a reduction of contaminations and for a better reproducibility of the results. Moreover important steps to pay attention for in a 2D-LC analyses were: *i)* the freshness of the protein sample and *ii)* the reproducible pH gradients performed by the exchange buffers. Protein extracted from different plant species and tissues (*Arabidopsis thaliana*; *Thlaspi caerulescens*; lichens, poplar) treated or not with different metals and metalloids such as Cs, Ni, Cd were analysed by 2D-LC methodology and data obtained enriched the information present in literature on 2D-gels giving a more comprehensive analysis of the plants proteome in the tested conditions.

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3.19

COMPARATIVE ANALYSIS OF PROTEOME CHANGES INDUCED BY THE TWO SPOTTED SPIDER MITE *TETRANYCHUS URTICAE* AND METHYL JASMONATE IN CITRUS LEAVES

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Citrus plants are currently facing increasing biotic and abiotic stresses and consequently, the characterization of molecular traits involved in the response mechanisms could facilitate selection of resistant varieties. To this end proteomics could be an useful tool to complement genomic and transcriptomic studies. However a few are proteomic studies on the effect of environmental stress in citrus plants. In this work, using two-dimensional gel electrophoresis, significant changes in the expression of several proteins extracted by *Citrus clementina* leaves infested by the two-spotted spider mite *Tetranychus urticae* and/or treated with methyl jasmonate were detected by image gel analysis. 50 protein spots were excised by gels and subjected to liquid chromatography mass spectrometry/tandem mass spectrometry to identify candidate proteins. The identified proteins were related to energy and primary metabolism, response to oxidative stress, and defence. Three proteins unknown in database were also detected. Furthermore, the mRNA expression of three defence–related proteins, a lectin-like, miraculin-like and acidic chitinase were assayed by RT-PCR in spider mite infested, methyl jasmonate treated or mechanically wounded leaves. RT-PCR data indicated different regulation of the expression as a function of different treatments. In summary, this paper reports for the first time the expression at protein level of defence-related proteins triggered by a mite attack in citrus leaves, and indicates that a proteomic approach.

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3.20

ACTIVITY STAINING OF SUPEROXIDE DISMUTASE AFTER TWO DIMENSIONAL GEL ELECTROPHORESIS IN PLANTS UNDER OXIDATIVE STRESS

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Environmental stress causes considerable loss of productivity in many crops. Plants have developed complex regulatory mechanisms in adapting to various environmental stresses. An increase in the production of reactive oxygen species (ROS) is a typical event occurring during different stress conditions and activating conflicting responses in plants.
The response of plants to stress is based on the transcriptional activation of a plethora of putative defence proteins and among them the enzymes involved in scavenging oxygen radicals play a major role [1]. The aim of this work is to investigate the role of antioxidant enzymes in counteracting ROS generated by osmotic, soil salinity and wounding stress. In this view the antioxidant enzyme expression patterns at protein and transcript level were investigated in leaves of *Citrus lemon* by comparative 2D electrophoresis tandem mass spectrometry and RT-PCR. The activity level of several antioxidant enzymes, among them SOD, was also assessed optimizing an "in-gel" activity staining technique after 2-D gel electrophoresis. The results show distinct regulation of antioxidant enzymes depending on different ROS generating stresses.

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4.1

2D-DIGE AND PATHWAY ANALYSES OF HEP-2 CELLS TREATED WITH THE ANTI-TUMOR DRUG VORINOSTAT AND GEFITINIB, A SELECTIVE EGFR TYROSINE KINASE INHIBITOR

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In this study, we focused our attention on the effect of histone deacetylase (HDAC) inhibitor vorinostat on head and neck squamous cell carcinoma (SCCHN). SCCHN are a heterogeneous class of tumors with poor prognosis and few effective treatment options, other than palliative chemotherapy in recurrent/metastatic setting. Since the epidermal growth factor receptor (EGFR) is involved in the development and progression of SCCHN, a more complete understanding of vorinostat combined with the selective epidermal growth factor receptor (EGF-R) tyrosine kinase inhibitor gefitinib should further improve the number of therapeutic options available for patients with SCCHN.

2D-DIGE was applied to analyze cellular extracts from HEP2 cells untreated and treated for 24 h with either vorinostat or gefitinib alone or with vorinostat/gefitinib combination. The DeCyder software (v. 7.0, GE Healthcare) analysis was performed and numerous differentially expressed proteins were visualized between the four settings examined. According to MALDI-TOF MS and ESI-Ion trap MS/MS several differentially expressed proteins were identified, and for some of them the proteomic analysis was validated by Western blotting. Finally, a pathway analysis of experimental data was performed by MetaCore and relevant networks were visualized in which proteins with drug-dependent level of expression were involved. In conclusion, our data proved the proteomic approach to lead a comprehensive analysis of proteins regulated by vorinostat and gefitinib, singularly or in combination, and to rise the possibility to find out and characterize essential therapeutic targets.

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4.2

IDENTIFCATION OF RPTPα AS RESPONSIBLE FOR FYN ACTIVATION IN PrP^C SIGNALLING

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Cellular prion protein (PrP^c) is a ubiquitous glycoprotein which physiological role is poorly characterized. It has been suggested that PrP^c participates in synaptic structure, neurite formation, copper metabolism, and signal transduction (1, 2). PrP^c antibody cross-linking stimulates the caveolin-dependent activation of Fyn leading to a rapid and transient phosphorylation of Erk1/2 (2, 3). The activity of Src family tyrosine kinases (SFK) is regulated by tyrosine phosphorylation at two sites, but with opposing effects. Phosphorylation of a tyrosine residue in the activation loop of the kinase domain upregulates enzyme activity, whereas phosphorylation by Csk of a tyrosine residue in the carboxy-terminal tail renders the enzyme less active stabilizing a noncatalytic conformation. An increasing number of protein-tyrosine phosphatases (PTPs) are being implicated in controlling SFK phosphorylation and activity. Such SFK-activating PTPs include both non-receptor (*e.g.* PTP1B, SHP1, and SHP2) and receptor PTPs (RPTPs, such as CD45, RPTPα, RPTPβ, and LAR) (4). Following this lead, we investigated the possibility that one of these PTPs is involved in PrP^c-dependent activation of Fyn. We peformed co-immunprecipitation experiments, PTPs in gel assay and immunoblotting assay with specific antibodies on isolated caveolae-like membrane domains. The analysis were conducted on 2D-electrophoresis gels. The results obtained with all these tests suggest RPTPα as major phosphatase involved in PrP^c signalling. This hypothesis was confirmed by cell stimulation experiments after siRNA-mediated RPTPα knock down.

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4.3

PROTEINS AND CARBONYLATION PATTERN IN TRAINED AND UNTRAINED RAT MUSCLES

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The mode, the intensity, the duration of the training and the intrinsic characteristics of the different skeletal muscles influence the way physical exercise affects the muscle. We investigated how high intensity aerobic training (10 wks treadmill at ~ 80% VO_{2max}) impinges on the formation of protein carbonyl adducts, a consequence of excess ROS production, in two opposite kinds of skeletal muscle, Anterior Tibialis (mostly fast glycolytic) and Soleus (mostly slow oxidative). In fact, during intense activity, the high rate of O_2 consumption in skeletal muscles can cause incomplete oxygen reduction, leading to the generation of ROS. ROS production may be quantitatively limited or counteracted by anti-oxidants, so that oxidative stress does not necessarily become established. These considerations promped us to examine the products of ROS damage. In particular, protein carbonylation is an irreversible reaction, whose amount reflects the intensity of the oxidative stress . The formation of carbonyl groups occurs either directly by reaction with ROS or indirectly by reaction with peroxidated lipids. The pattern of carbonylated proteins was studied in both control and exercised muscles by 2D-GE followed by Western Blot with anti dNTP antibodies. In order to minimize the biological variability between different individuals, the images of Western Blots were used to compose four synthetic gels in which only matched spots were present. This study enabled us to obtain the basal pattern of carbonylation characterizing the two types of skeletal muscles and to compare them with the exercise-induced variations in the carbonylation level of specific proteins.

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4.4

ELUCIDATION OF THE Cbx7 ROLE IN HUMAN CARCINOMAS

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Chromobox protein homolog 7 (CBX7) belongs to the chromobox family protein and it is a member of the Polycomb repressive complex 1 (PRC1), that maintains developmental regulatory genes in a silenced state. The analysis of CBX7 expression in a large number of thyroid carcinoma samples revealed a progressive reduction of CBX7 levels that is well related with the malignancy grade of the thyroid neoplasias and colon carcinoma. In order to investigate the mechanism by which the loss of CBX7 expression correlates with a highly malignant phenotype, we identified CBX7 interacting proteins by functional proteomic approaches. CBX7-V5 and its interacting proteins were immunoprecipitated with anti-V5 antibodies from nuclear extract of HEK 293 expressing the tagged protein. The protein components were fractionated on SDS-PAGE and identified by tandem mass spectrometry and protein database search. Among several others interactors, we selected histone deacetylase 2 (HDAC2), which is well known to play a key role in neoplastic cell transformation. We confirmed by coimmunoprecipitation that CBX7 physically interacts with the HDAC2 protein and is able to inhibit its activity. Then, we showed that both these proteins bind the E-cadherin promoter and that CBX7 up-regulates E-cadherin expression. Moreover, the expression of CBX7 increases the acetylation status of the histones H3 and H4 on the E-cadherin promoter. Therefore, the ability of CBX7 to positively regulate E-cadherin expression by interacting with HDAC2 and inhibiting its activity on the E-cadherin promoter would account for the correlation between the loss of CBX7 expression and a highly malignant phenotype [1].

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4.5

MAPPING THE ESTROGEN RECEPTOR BETA INTERACTOME OF BREAST CANCER CELL NUCLEI BY TANDEM AFFINITY PURIFICATION AND NANOLC-MS/MS

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Estrogens control genome activity by binding to Estrogen Receptor alpha and beta (ER-α and -β), that are members of the steroid/nuclear receptor superfamily of transcriptional regulators. ER ligands induce a change in receptor conformation that results in nuclear translocation, protein dimerization and binding to chromatin, where they assemble in large multiprotein complexes to control gene transcription [1-2]. In breast cancer cells this leads to the causal relationships between estrogen actions and the hormone-responsive tumor phenotype. The two ERs share a conserved modular structure but show strikingly different functional roles, for example in regulation of cell proliferation and gene transcription. Identification of functional protein-protein interactions involving ERβ is an useful approach to investigate the role of this receptor subtype in estrogen signalling and to identify different signal transduction pathways involving ERα and ERβ. To this aim, we applied a proteomics work flow based upon Tandem Affinity Purification, glycerol gradient sedimentation, nanoLC-MS/MS and data analysis, to isolate and identify ligand-activated ERβ specific partners in MCF-7 cells stably expressing exogenous human ERβ fused with a TAP-tag. This led to the identification of 303 molecular partners specifically isolated from TAP-ERβ expressing cells. A comparison of these results with a previously generated dataset of proteins specifically associated to ERα in the same cell compartment highlighted 160 proteins that associate only with ERβ, involved in distinct molecular functional and biological process with respect to ERα [3].

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4.6

IDENTIFICATION OF PROTEINS ASSOCIATED WITH LIGAND-ACTIVATED ESTROGEN RECEPTOR ALPHA IN HUMAN BREAST CANCER CELL NUCLEI

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Estrogen receptor alpha (ERα) is a key mediator of estrogen actions in breast cancer cells. This nuclear receptor acts as transcription factor that, once bound to the hormone, undergoes structural changes resulting in its nuclear translocation and docking to specific chromatin sites [1]. In the nucleus ERα assembles in multiprotein complexes acting as final effectors of the genomic estrogen signalling through chromatin remodelling and epigenetic modifications, leading to dynamic and coordinated regulation of hormone-responsive genes. Understanding the effects of ligand-activated ERα in target cells requires identification of the molecular partners acting in concert with this nuclear receptor to transduce the hormonal signal. Tandem Affinity Purification, glycerol gradient centrifugation and MS analysis were applied to isolate and identify proteins interacting with ligand-activated ERα in MCF-7 cell nuclei. Cell clones expressing human ERα fused to a Tandem Affinity Purification-tag were generated [1] and used to purify native nuclear ER-containing complexes by two sequential steps of affinity chromatography [2-3]. This led to the identification of 264 ER-associated proteins, whose functions highlight the hinge role of ERα in coordination of multiple hormone-regulated nuclear processes in breast cancer cells [3]. Among these, a hormone-regulated dynamic complex comprising β-actin, myosins and several proteins involved in actin filament organization and dynamics was isolated. Such proteins are known to participate in actin-mediated regulation of gene transcription, chromatin dynamics and ribosome biogenesis. Gene knock-down experiments show that gelsolin and the nuclear isoform of myosin 1c are key determinants for assembly and/or stability of these nuclear network [2].

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4.7

CHROMATIN PROTEOMICS: SILAC-BASED QUANTITATIVE METHODS TO PROFILE HISTONE POST-TRANSLATIONAL MODIFICATIONS DYNAMICS *IN VIVO*

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In living cells, the N-terminal tails of core histones are subjected to a range of covalent post-translational modifications that modulate chromatin structure and function[1]. Histone methylation has been long considered an epigenetic mark which mediates the inheritance of chromatin functional states, basing on the assumption of its irreversibility. The discovery of de-methylases revolutionized this widely accepted concept suggesting the reversibility of methylation. As such, an approach for the determination of *in vivo* histone methylation is of tremendous biological importance, though extremely challenging.

We describe heavy methyl SILAC strategy as a novel proteomics approach accomplishing this daunting task. In hmSILAC methylation is isotope-labeled, resulting in high confidence detection and quantification of methylations *in vivo*[2]. We performed time course experiments in HeLaS3 cell lines and in murine embryonic fibroblast derived from mice double null for the enzymes Su(var)4-20 and Su(var)3-9. Methylation kinetics where simultaneously determined for several methylation sites involved in both transcription activation (H3K4, H3K36, H3K79), as well as repression (H3K9, H3K27, H4K20): mono-methylation had the fastest heavy methyl incorporation, with no significant difference among sites. Instead, di-, tri- methylation states showed a distinct kinetics between sites/marks functionally linked to either gene activation or gene repression, with ”active” sites having significant faster turnover. Studies on Su(var)4-20 knock-out showed the expected drop in H4K20me2 and H4K20me3, with increase in H4K20me, together with a reduction in the kinetics of the mono-methylation reaction. Parallel results for Su(var)3-9 mutant will be described.

This innovative approach provides a tool to measure histone methylation kinetics *in vivo,* with a major impact upon epigenetic research.

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4.8

PED REGULATES CELL MIGRATION/INVASION PROCESSES IN HUMAN NON SMALL CELL LUNG CANCER CELLS THROUGH RAC1

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PED (phosphoprotein enriched in diabetes) is a 15 KDa protein involved in many cellular pathways and human diseases including type II diabetes and cancer, senescence, autophagy, proliferation and migration. PED function is regulated by phosphorylation on two different serine residues: Ser104, phosphorylated by PKC (Protein Kinase C), and Ser116, phosphorylated by AKT/PKB (Protein Kinase B) and CamKII.

We recently reported that PED is over expressed in several human cancers and mediates resistance to induced apoptosis. To better understand its role in cancer, we focused on PED interactome in non small cell lung cancer (NSCLC). Using the Tandem Affinity Purification (TAP), among several PED interactors, we identified Rac1, a member of mammalian Rho GTPase protein family. Rac1 regulates a wide range of cellular effects such as secretory processes, phagocytosis of apoptotic cells, epithelial cell polarization, growth factor-induced formation of membrane ruffles, ROS generation, lamellipodia formation and cell migration.

We showed that PED coadiuvates Rac1 activation by regulating AKT mediated Rac1-Ser71 phosphorylation. Furthermore, the expression of constitutively active Rac affected PED-Ser104 phosphorylation, which is important for ERK 1/2 nuclear localization mediated by PED. Through specific Rac1-siRNA or its pharmacological inhibition, we showed that PED augmented migration and invasion in Rac1-dependent manner in non small cell lung cancer cells. In conclusion, we showed for the first time that PED and Rac1 interact and that this interaction modulates cell migration/invasion process through ERK1/2 pathway. This protein might result, therefore, to be a good target for anti neoplastic therapy [1].

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4.9

EXPLORING THE BIOCHEMICAL MECHANISMS OF CYTOTOXIC GOLD COMPOUNDS: A PROTEOMIC STUDY ON A2780 SENSITIVE AND RESISTANT CELLS

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We have recently shown that a group of structurally diverse gold compounds are highly cytotoxic toward a panel of 36 human tumor cell lines through a variety of biochemical mechanisms [(1)]. A classic proteomic approach is exploited here to gain deeper insight into those mechanisms. This investigation is focused on Auoxo6, a novel binuclear gold(III) complex, and auranofin, a clinically established gold(I) antiarthritic drug. First, the 72-h cytotoxicity profiles of Auoxo6 and auranofin were determined against A2780 human ovarian carcinoma cells (cis-platin sensitive and resistant). Subsequently, protein extraction from gold-treated A2780 cells (cis-platin sensitive and resistant) and 2D gel electrophoresis separation were carried out according to established procedures. Notably, both metallodrugs caused relatively modest changes in protein expression in A2780 sensitive cells in comparison with controls as only 11 out of approximately 1,300 monitored spots showed appreciable quantitative changes. Very remarkably, six altered proteins were in common between the two treatments. Eight altered proteins were identified by mass spectrometry; among them was ezrin, a protein associated with the cytoskeleton and involved in apoptosis. Interestingly, two altered proteins, i.e., peroxiredoxins 1 and 6, are known to play crucial roles in the cell redox metabolism. Increased cleavage of heterogeneous ribonucleoprotein H was also evidenced. Overall, the results of the present proteomic study point out that the mode of action of Auoxo6 is strictly related to that of Auranofin and that the induced changes in protein expression are limited and selective. We are using the same criteria to study the proteome of the gold-treated A2780 resistant cells.

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4.10

FUNCTIONAL INSIGHT OF TACE/TIMP3 DYAD IMPACT IN CHRONIC METABOLIC-INFLAMMATORY DISEASES

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Quantitative proteomics is widely used for examining differences in global protein expression between biological samples in order to elucidate fundamental mechanisms of the molecular processes under study. In this scenario, large-scale quantitative shotgun tandem mass spectrometry coupled with a broader statistical and bioinformatic framework serves as a flexible proteomic platform for a wide systematic analysis of the investigated biological issue.
We report on a proteomic study of different genetic mice models under metabolic stress to identify proteins linked to the biological functions of the sheddase TACE and its inhibitor Timp3 in homeostasis and inflammatory disease states. In fact, the regulation of TACE plays a major a role in the pathogenesis of insulin resistance and vascular inflammation associated with obesity [1, 2].
We analysed different mice models (WT, Timp3 KO, Timp3 transgenic, LDLR KO), different tissue (liver, kidney, wat) and different metabolic stress (High fat diet, Streptozocin).
Our high-ending mass spectrometry investigations were performed by a label-free quantitative shotgun proteomic experiments on a Q-Tof mass spectrometer based on nano ultra performance liquid chromatography (nUPLC) coupled to MS^E. This proteomic strategy allowed us to perform multiplexed comparisons enabling highlighting of cellular protein abundance changes and proteins dosage profiling unique to TACE/Timp3 dyad activity. The final bioinformatic integration and interpretation of all the experimental data acquired from multiple shotgun proteomics measurements elucidated molecular metabolic and signalling pathways to reconstruct the sequence of molecular events that ultimately are responsible for the TACE/Timp3 dependent specific phenotype.

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4.11

GLYCOPROTEOME AND PHOSPHOPROTEOME SIGNALING DURING MOUSE BRAIN DEVELOPMENT

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Background: The precise orchestration of several temporally and spatially regulated stimuli is required for proper mammalian brain development. Neuronal stimuli transduce into molecular changes of downstream effectors that undergo to a series of post-translational modifications (PTMs) such as phosphorylation and glycosylation. The increasing evidences for cell surface glycan remodeling and intracellular signaling needs a site-specific enrichment method for identification and quantification of PTMs at the same time. We have used the high affinity of TiO_2 chromatography towards negatively charged molecules such as sialic acid (SA) containing glycopeptides [1] and phosphopeptides [2] in combination with isobaric tags (iTRAQ) and hydrophilic interaction liquid chromatography (HILIC) to quantitatively analyze the regulation of cell surface sialoglyco and phopshoproteins during brain development. **Methods:** Membrane proteins from postnatal 0, 8, 21 and 80 days mouse brains were extracted, digested with trypsin and 4-plex iTRAQ^{TM} labeled. Glycopeptides and phosphopeptides from a total of 400 ug of peptides were enriched using TiO_2 chromatography. The eluate was deglycosylated with PNGase F and the deglycosylated-peptide/phosphopeptide mixture was fractionated using HILIC and analyzed using RPLC-MS/MS. **Results:** We have identified and quantified 1958 non-redundant sialylated *N*-linked glycosylation sites mapping 1054 glycoproteins and 5868 non-redundant phosphorylation sites representing 3211 phosphoproteins from the membrane fraction, showing the possibility of performing comprehensive analysis of PTMs on tissue using low sample amount. The results allowed us to get a deeper understanding of the molecular processes during brain development and in particular the selected pathways triggered during neuronal progression. Indeed, cluster and pathway analysis revealed the activation of axon guidance signaling in early development and synaptic potentiation, related to learning and memory process, during later development. In many of the clusters we identified novel uncharacterized proteins potential relevant for learning and memory and brain development.

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4.12

INHIBITION OF HEPATIC CANCEROGENESIS BY ROSIGLITAZONE: EVIDENCES OF A PPARGAMMA-INDEPENDENT MECHANISM THROUGH THE DOWN-REGULATION OF RUVBL-1

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Rosiglitazone (RGZ) belongs to the Thiazolidinediones (TZDs) class of insulin-sensitizer for the treatment of type II diabetes and is a potent agonist of the nuclear receptor Peroxisome Proliferator-Activated Receptor gamma (PPARgamma). TZDs have been reported to inhibit cell growth and promote differentiation in several epithelial cancer cell lines including liver. We previously reported that administration of RGZ to transgenic mice prone to HBV-related hepatocellular carcinoma inhibits tumor formation. In this model, we identified by proteomic analysis (2D-DIGE) Ruvbl-1 as one of the proteins downregulated by RGZ administration. Ruvbl-1 is an ATP-dependent helicase involved in many cellular processes including gene expression, cell growth, apoptosis and cancer.

In order to asses the role of Ruvbl-1 in RGZ inhibition of HCC cell growth, we have carried out a set of experiments, both on mouse (Hepa1-6) and human (HepG2) liver cancer cells. We have shown that RGZ inhibition of human and mouse HCC cell proliferation is possibly mediated by a down-regulation of Ruvbl-1. Our findings explicitly indicate that PPARgamma activation do not mediate RGZ inhibition of Ruvbl-1. Ruvbl-1 is upregulated in human HCC tissue and its inhibition may represent an interesting anti-tumoral target. Future studies will be conducted to define the molecular mechanism by which RGZ inhibits Ruvbl-1 expression and Hepatic Cancer Cell growth.

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4.13

INFLUENCE OF PHOSPHORYLATION STATE ON THE ACTIVITY OF TRANSLATION INITIATION FACTOR EIF4E

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In metazoa, the spatio-temporal translation of diverse mRNAs is essential to guarantee proper oocyte maturation and early embryogenesis. The eukaryotic translation initiation factor 4E (eIF4E), which binds the 5' cap structure of eukaryotic mRNAs, associates with either stimulatory or inhibitory factors to modulate protein synthesis [1].
eIF4E in human is phosphorylated on Ser209 which corresponds to the Ser251 in the Drosophila protein. The eIF4E phosphorylation state changes in response to extracellular signals and its influence on the translational activity is still unclear.
In order to investigate the relevance of the phosphorylation state of eIF4E on the translation initiation process, Drosophila mutants expressing the protein eIF4ESer251Ala, where the phosphorylation site was suppressed, were obtained. These mutants are smaller than wild type Drosophila, demonstrating that the eIF4E phosphorylation state is important for the correct development. A functional proteomic analysis based on the isolation of eIF4E protein complexes by affinity chromatography on m7GTP-Sepharose beads was performed. The protein components were separated by SDS/PAGE and identified by LC-MSMS. We analysed both Drosophila wild type and eIF4E^{Ser251Ala} mutants.
The number of interacting protein identified was largely lower than the number of wild type protein interactors. In particular several proteins involved in the translation activation process were absent in the mutant interactome, suggesting a molecular correlation between the genotype and the phenotype.
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4.14

CONFORMATIONAL TRANSITIONS OF INTRINSICALLY DISORDERED ARCHITECTURAL TRANSCRIPTION FACTORS HMGA. NEW INSIGHTS BY MASS SPECTROMETRY.

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Intrinsically disordered proteins, able to modulate their binding properties in respect to the nature and conformation of the partner they interact with, are often highly connected hubs orchestrating numerous and different cellular networks. Oncofetal architectural transcription factors HMGA belong to this peculiar class of flexible proteins playing a central role in several nuclear functions, interacting with DNA, RNA, and proteins and being modulated in their activities by numerous and different PTMs. A constitutive and multiple phosphorylation affects C-terminal tail of HMGA, suggesting a role of this domain in the determination of protein functions, especially considering that truncated forms, lacking the acidic tail, display an increasing cellular oncogenic potential, if compared with wild type proteins.
Performing *in vitro* enzymatic assays we demonstrated that C-terminal truncated forms and wild-type proteins behave as strongly different substrates. In fact truncated HMGA are much more susceptible towards the activity of kinases and methyl-transferases known to modify HMGA proteins, probably due to their different accessibility. ESI-MS and IM-MS analyses underlined the presence of different levels of compactness for HMGA natively disordered proteins, ranging from a completely denatured and extended status to a molten-globule like conformation. Ion Mobility Mass Spectrometry data moreover showed that full length proteins have a more compact conformation in respect to truncated ones and that this compactness is enhanced when C-terminal phosphorylation is present. Acidic tail and its phosphorylations seem therefore to be involved in masking the accessibility of the inner portions of the protein, thus affecting HMGA PTMs status, and therefore their activity.

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4.15

EFFECT OF SCO1 DELETION ON *SACCHAROMYCES CEREVISIAE* METABOLISM

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The Sco proteins are mitochondrial metallochaperone proteins with essential roles in copper delivery to cytochrome *c* oxidase. Biochemical studies of the yeast proteins demonstrated specific transfer of copper from Cox17p to Sco1p, and physical interactions between the Sco proteins and Cox2p. The deletion of the yeast *SCO1* gene results in a respiratory deficient phenotype. The present study aimed at gaining a more detailed insight into the effects of SCO1 deletion and thus COX deficiency on *Saccharomyces cerevisiae* metabolism.

We performed a comparison, using 2-D electrophoresis, between the protein pattern of Δ*SCO1*-mutants and Wild Type BY4741 grown in both, a fermentable and non-fermentable carbon source. Five different gels, resulting from different growths, were produced for each of the four different conditions.

The 93 differentially expressed spots resulted from the image analysis have been grouped in order to follow the expression profile of these variations in all our tested conditions.

Interesting, the expression profiles of 43 variations are very similar in both the fermentative conditions, and Δ*SCO1*-mutants grown in 3% glycerol but different in the Wild Type BY4741 grown in a non-fermentable carbon source. Among these proteins we found: Enolase 2, Pyruvate decarboxylase 1, Peroxiredoxin Type-2, Thioredoxin reductase 1 and Glyceraldehyde-3-phosphate dehydrogenase**.** Moreover 12 protein species, such as Alchol dehydrogenase 1, are considerably overexpressed in Δ*SCO1*-mutants grown in a non-fermentable medium compared with the tree other conditions.

In conclusion the proteomic analysis demonstrated that on a non-fermentable medium the loss of Sco1p was accompanied by an increased level of fermentative and antioxidant enzymes.

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4.16

MODULATION OF THE PROTEOMIC PROFILE IN HEPATOCYTES BY CONJUGATED LINOLEIC ACID

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Conjugated linoleic acid (CLA), a fatty acid present in ruminant meat and dairy products, is a mixture of positional and geometrical isomers (CLA c9,t11 and t10,c12) of linoleic acid. It was recently proven that CLA had beneficial effects on human health. Since lipid metabolism appears to be of pivotal relevance in CLA beneficial effects, along with the modulation of gene expression in liver, muscle and adipose tissue, we decided to investigate the ability of CLA to modulate proteomic profile in animal tissues.

The first step of this study was to asses the modification of protein expression in liver. We used hepatocytes isolated from fasting male Wistar rat, incubated at 37°C for 1 hour with a 10 μM CLA mixture; as a control fatty acid the oleic acid was used. The proteomic profile was analyzed using two dimensional electrophoresis (2DE). Differences in proteins expression of CLA treated hepatocytes were assessed by PDQuest software. A certain number of proteins, included in a 10-37 KDa weight range and in a 4-8 pH range, showed a differential expression profile. Spots corresponding with these proteins were purified, digested with trypsin and identified by mass spectrometry. The present investigation demonstrates that CLA is able to modify the proteomic profile of rat hepatocyte. Moreover we further investigated the effect of the specific isomers (CLA c9,t11 and CLA t10,c12) present in the CLA mixture on the proteomic profile of the hepatocyte.

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4.17

TOWARD THE UNDERSTANDING OF CORNELIA DE LANGE SYNDROME THROUGH A PROTEOMIC APPROACH

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Cornelia de Lange syndrome (CdLS) is a rare genetically heterogeneous disorder characterized by distinctive dysmorphic facial features, upper limb abnormalities, growth retardation and neurodevelopment delay, from moderate to severe.

Mutations in two cohesin structural components SMC1A or SMC3 (Structural Maintenance of Chromosomes 1A and 3) contribute to the 5% of CdLS cases and result in a mild phenotype.

The current study was the first proteomic approach to analyse the Cornelia de Lange Syndrome. We have compared lymphoblastoid cell lines from CdLS patients affected by different mutations in SMC1A and SMC3 genes with healthy control individual cells, using the quantitative 2D-DIGE analysis. Several differences were successfully identified using MALDI-TOF MS and ESI-IonTrap MS. Several proteins with defective expression in CdLS patients were found to be involved in important cellular processes such as metabolic pathways, protein folding, response to stress and cytoskeleton remodeling.

Network analysis, followed by Western blot validation, indirectly confirmed the reliability of proteomic data and suggested a possible role for c-Myc proto-oncogene in the pathogenesis of CdLS. Further investigation will be applied to better understand how the identified proteins may provide new light on the functional role of cohesin subunits and their implication in Cornelia de Lange Syndrome.

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4.18

EFFECT OF ACETYL-L-CARNITINE CHRONIC ADMINISTRATION TO OLD RAT ON THE LIVER MITOCHONDRIAL PROTEOME

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Acetyl-L-carnitine (ALCAR) is a biomolecule able to limit age-linked mitochondrial decay [1] and to activate mitochondrial biogenesis in skeletal muscle [2]. The aim of this work was to study the effect of *in vivo* ALCAR administration to old rats on the liver mitochondrial proteome.

Experiments were performed on three groups of male Fisher-344 Charles-River rats: 12-month-old rats (adult); 28-month-old rats (old); ALCAR-treated old rats (treated). Old rats were treated with ALCAR daily dose of about 0.5 g/kg of body weight for two months. Mitochondria were prepared by differential centrifugation from freshly excised livers and mitochondrial proteins were separated in first dimension on 18 cm 4-7 and 6-11 pH gradient IPG strips and in second dimension on 10% SDS polyacrylamide gels. Differentially expressed proteins were identified by MALDI and nano ESI MS/MS mass spectrometry.

In old rats *versus* adult rats, 45 differentially expressed proteins were identified, the 66% of these proteins belong to metabolic mitochondrial metabolisms. Several proteins were altered in ALCAR-treated rats *versus* old rats and in particular ALCAR treatment reverted the age-related alteration of 16 proteins. Previously we demonstrated that, in liver, ALCAR treatment to old rats reduces the accumulation of the overoxidized form of Peroxiredoxin III, a mitochondrial antioxidant enzyme [3] and activates the PGC-1α-dependent mitochondrial biogenesis pathway reduced with age [4]. These results suggest that ALCAR induces mitochondrial biogenesis and counteract the age-related alterations of several mitochondrial proteins in liver.

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4.19

STUDY OF REDOX REGULATION OF OGG1 ACTIVITY BY MASS SPECTROMETRY.

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One of the major DNA modifications caused by reactive oxygen species (ROS) is the premutagenic lesion 7,8-dihydro-8-oxoguanine. OGG1 is the major DNA glycosylase/AP lyase able to repair these lesions in eukaryotes. ROS may also target OGG1 itself, decreasing its activity. This effect is reversible in the presence of reducing agents, suggesting the involvement of disulphide bond(s) not yet characterized.
The serine-to-cysteine polymorphism at position 326, frequent in humans, renders OGG1 extremely sensitive to oxidation, which impairs its DNA glycosylase activity both *in vitro* and *in vivo* (Bravard et al). Thus, the analysis of the redox state of cysteines in wild-type and polymorphic OGG1 is crucial to understand the mechanism underlying oxidation sensitivity.
In this work, we found that polymorphic—but not wild-type—OGG1 forms dimers that can be dissociated by reducing agents. By enzymatic digestion and mass spectrometry analysis we were able to characterize several disulphide bonds whose formation depends on the sequence of the OGG1 protein and the oxidative conditions. In particular, in the polymorphic OGG1, cysteine 326 is able to form several alternative intra-molecular disulphide bonds. In oxidative conditions, both the wild-type and the polymorphic proteins form additional disulphide bonds in the N-terminal region, but the polymorphic OGG1 shows one more specific bond between cysteines 28 and 326. We are currently determining in which structure OGG1 is able to bind DNA and exert enzymatic activity.
This study highlights previously unknown OGG1 structure–function relationships and their modulation in the presence of oxidative stress.

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4.20

PROTEOMIC ANALYSIS OF "LIPID RAFT" IN ENDOTHELIAL PRECURSOR CELLS

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Angiogenesis, the formation of new blood vessels, has long been considered to result from pre-existing vessels. Identification of endothelial progenitor cells (EPCs) in peripheral blood has highlighted an alternative angiogenesis mechanism based on EPC recruitment from bone marrow. EPCs are able to migrate, proliferate, differentiate into mature endothelial cells ("postnatal vasculogenesis"). In our laboratory we isolated a type of EPC termed "Endothelial Colony Forming Cells" (ECFC), characterized by high proliferative potential, colony formation upon replating, and de novo blood vessels formation in vivo. Lipid cell membrane composition is critical to cell biological functions, including angiogenesis. "Lipid rafts" are dynamic micro-domains of the cell membrane, rich in cholesterol, sphingolipids, glycolipids, trans-membrane protein receptors, integrins and signalling molecules. Lipid raft composition of EPCs is still unknown, therefore, the study their composition can provide important information on EPC-dependent angiogenesis. Many methods are available to modify the lipid rafts composition and consequently the behaviour of the cells. We used methyl-β-cyclodextrin (MCD) which induce cholesterol depletion. In this study we analyzed the proteomic profile of lipid rafts ("raftosome") of ECFC, before and after treatment with vascular endothelial growth factor (VEGF), which has a fundamental role in angiogenesis. In particular proteins from the lipid raft fractions were separated by 2D-GE and differences between VEGF treated cells and control cells were evedentiates. Furthermore the 2D-GE profile of the fractions corresponding to the "non–raft" portion of the cellular membrane were analysed evidencing a pattern of proteins deeply different from that one of the lipid raft.
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4.21

SERUM GLYCOPROTEOMICS OF A NOVEL GENETIC GLYCOSYLATION DISEASE (DPM2 MUTATION)

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Mass spectrometry (MS) based glycoproteomics of human serum is crucial in discovery and characterization of congenital disorders of glycosylation (CDG) [1], genetic diseases that affect the assembly and/or processing of glycans on glycoproteins (CDG type I and II, respectively). CDG have a variable clinical spectrum which ranges from multisystem to single-organ diseases, with almost 40 types identified as yet [2].

We set up a multiplexed analysis of human serum by MALDI-TOF MS to analyse, in the same sample, both native glycoprotein glycoforms and N-glycan profile [3]. By this approach, we could be able to characterize distinct underglycosylation profiles in patients with CDG type I or type II defects. We report on clue MS glycoproteomic findings for the detection of a novel CDG type identified in a Sicilian child with prominent neurological involvement and fatal disease course. In this patient, MALDI spectra of native serum Transferrin, showed a distinct underglycosylation profile with a main normal di-glycosylated Tf and an additional glycoform owing to mono-glycosylated Tf.

Unlike CDG-Ia (PMM deficiency), no a-glycosylated species were detectable. Thus, MS glycosylation analyses in this patient pointed to a different primary defect than that observed in CDG-Ia. In order to ascertain a generalized defective N-glycosylation, we furthermore studied and defined by MALDI analysis hypoglycosylation profiles also in serum alpha-1-antitrypsin. Demonstration of dol-PP-GlcNAc2-Man5 precursor accumulation in patient's fibroblasts and molecular analyses of genes encoding dolichol-phosphate-mannose (DPM) synthase complex subunits, lead to detection of a novel condition due to mutation in DPM2 subunit gene. This is the first glycoproteomic characterization of a patient with CDG due to DPM2 gene mutations.

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5.1

PROTEOMIC AND METABOLOMIC CHARACTERIZATION OF HAZELNUT VARIETIES TYPICAL OF REGIONE CAMPANIA

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Hazelnut (*Corylus avellana L.*) seeds are largely used in a range of confectionery products and contain many well-characterized allergens. Hazelnut is a common cause of food induced allergic reaction. Several hazelnut allergens with different relevance have been identified and characterized up to now [1]. It is very important to set up methods able to detect any protein modifications resulting from food processing, able to reduce allergenicity. Is know that the roasting process reduces the probability of the development of allergy in sensitive subjects by abolishing IgE binding to the protein Cor a 1 [2].

The aim of the present study was to achieve a detailed and comprehensive characterization of hazelnut cultivars autochtonous of Regione Campania (Tonda from Lauro, San Giovanni and Mortarella) using a combined approach based on proteomic and metabolomic strategies. The protein fraction was separated by RP-HPLC, mono- and two-dimensional PAGE and analysed by mass spectrometry for tryptic peptide fingerprinting. Modifications induced by the roasting treatments were also studied. We played particular attention to the potentially allergenic proteins of the different hazelnut varieties, in order to identify cultivars with the best safety characteristics for allergic persons.

In order to obtain a more complete varietal characterization on a molecular basis, a metabolomic approach was also applied on the volatile secondary metabolites, whose detection was carried out by means of HS-SPME-GC/MS analysis. This allowed identification of molecular tracers of the different varieties in transformed products (creams, chocolate, ice-creams, cake fillings), providing useful information from both a technological and safety point of view.

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5.2

INTERACTION BETWEEN PROTEINS OF PLANT ORIGIN AND WINE COMPONENTS: MOLECULAR-BASED CHOICE OF PROTEIN FINING AGENTS FOR ORGANOLEPTIC IMPROVEMENT

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Gelatine, casein, egg albumin, and, more recently, proteins of from plant sources are commonly used in winemaking as fining agents to remove particles responsible for turbidity, to improve stability, and to control browning, over-oxidation, and bitterness during ageing. The formation of covalent and non-covalent interactions between the protein matrix and wine polyphenolics is the basis of the flocculation and of the consequent clarification which results in an overall improvement of wine quality parameters. In this work we studied the molecular basis of the interactions between plant proteins (soy, gluten, lentil and pea proteins) and polyphenolic compounds responsible for organoleptic as well as stability properties of wines, by using mass spectrometry methodologies (LC-ESI MS, MALDI TOF MS). Protein surface hydrophobicity was investigated in wine-like model system by spectrofluorimetric determination of changes in the binding properties of 1,8-anilinonaphthalenesulfonate (ANS), used as extrinsic fluorescent probe [1]. Hydrophobic interactions between phenolic compounds and protein finings were evaluated by the study of competition of phenolic compounds with the ANS probe for the same binding sites [2]. Structural characterization of phenolic compounds (polymer chain length and chemical structure and composition of individual chains), as well as their interactions with the plant proteins, essential for the definition of protein binding affinity, was performed by means of mass spectrometry techniques. Differences among interactions between polyphenols with the various protein matrices have been related with the quality parameters of the resulting wines.

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5.3

HUMAN MILK PROTEINS: AN INTERACTOMICS AND UPDATED FUNCTIONAL OVERVIEW

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Milk and milk fractions are characterized by a wide array of proteins, whose concentration spans across several orders of magnitude. By exploiting a combined approach based on functional gene ontology enrichment (FatiGO/Babelomics), hierarchical clustering, pathway and network analyses, we merged data from literature dealing with protein-oriented studies on human milk. A total of 285 entries defined a non-redundant list upon comparison with the Ingenuity Knowledge Base from the Ingenuity Pathway Analysis software. Results were compared with an inventory of bovine milk proteins gathered from dedicated proteomic studies. A protein core of 106 proteins was found, with most of the entries associated to three main biological functions, namely nutrient transport/lipid metabolism, concretization of the immune system response and cellular proliferation processes. Our analyses confirm and emphasize that the biological role of the human milk proteins is not only limited to the provision of external nutrients and defense molecules against pathogens to the suckling, but also to the direct stimulation of the growth of neonate tissues/organs and to the development of a proper independent immune system, both through the induction of a number of molecular cascades associated to cell proliferation/differentiation. The latter aspects were previously investigated by single-molecule dedicated studies, missing the holistic view that results from our analysis.

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5.4

BOVINE MILK PROTEINS: AN UPDATED AND REFERENCED OVERVIEW THROUGH PATHWAY, NETWORK AND GO TERM ENRICHMENT FOR BIOLOGICAL AND MOLECULAR FUNCTIONS

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Bovine milk undoubtedly represents a valuable resource of nutrients for the lactating calf, and a key nutrient for human consumption for centuries. This is the main reason why wealth of data are present in litearature dealing with massive proteomics studies on milk fractions and independent targeted studies on specific proteins (or groups of proteins, e.g. hormones, cytokines). In this article, we merged data from literature to compile a referenced and annotated list of 574 non-redundant entries. The list was exploited for integrated *in silico* analyses, including functional GO term enrichment (FatiGO/Babelomics), multiple pathway and network analyses. As expected, most of the proteins could be regrouped under pathways/networks/ontologies referring to nutrient transport/lipid metabolism and objectification of the immune system response. Notably enough, a third group of functions was individuated as the most statistically significant one, which included proteins involved in induction of cellular proliferation processes, anatomical and hematological system development. Although this latter function of bovine milk proteins has long been postulated, articles reported so far mainly focused on a handful of molecules. Hereby we provide an integrated and in-depth overview.

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5.5

OXIDATIVE DAMAGE INDUCED BY HERBICIDES IS MEDIATED BY THIOL OXIDATION AND HYDROPEROXIDES PRODUCTION

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Toxicological and environmental issues are associated with the extensive use of agricultural pesticides, though the knowledge of their toxic effects as commercial formulations is still far from being complete [1,2]. In this work, we have investigated the impact of three herbicides as commercial formulations, namely Pointer, Silgif, and Proper Energy, on some oxidative parameters of a wild type *Saccharomyces cerevisiae* strain such as glutathione content, hydroperoxides production, and protein oxidation. Particularly, we investigated protein thiol oxidation, as thiols should be considered critical components in redox systems biology, regulating through their reversible/irreversible oxidation many biological functions [3]. We combined selective labelling of cysteine residues with two-dimensional electrophoresis (2D-PAGE) to evaluate yeast protein oxidation induced by herbicide stress. Being yeast a well-established model of eukaryotic cells, especially as far as it regards the stress response, our results may be indicative of potential damages on higher eukaryotes. We found that herbicide-mediated toxicity towards yeast cells could be the result of an increased production of hydroperoxides (like in the case of the herbicides Pointer and Silglif) or advanced oxidation protein products and lipid peroxidation (especially in the case of the herbicide Proper Energy). Through a redox-proteomic approach we found also that, besides a common signature, each herbicide showed a specific pattern for protein thiols oxidation. Our results indicate that herbicide-induced oxidative stress could impair metabolic fluxes of carbohydrates (thus eventually impairing the fermentative performance of wine yeast strains) as well as specific metabolic pathways.

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5.6

SURFOME ANALYSIS OF A WILD-TYPE WINE *SACCHAROMYCES CEREVISIAE* STRAIN

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The fungal cell wall is a dynamic structure protecting cells from osmotic and physical stresses and providing interactions with the environment. In *Saccharomyces cerevisiae* the cell wall undergoes profound changes during various cellular processes. Cell wall proteins are crucial components of this structure, and their expression and incorporation into the cell wall are temporally and spatially controlled [1]. In this work, we investigated through a mass spectrometry (MS)-based approach the cell surface-exposed proteins (surfome) in a wild type wine yeast strain, namely K310, during the fermentation process.
K310 cells were grown in a synthetic modified medium to get closer to the conditions of wine production and collected at the beginning and at the end of the fermentation. Cells were harvested, extensively washed and incubated at 37 °C for 30 min with trypsin. Then supernatants were filtered and submitted to MS. Before and after treatment with trypsin, the integrity of yeast cells was checked with Annexin V/propidium iodide assay.
The use of a MS-based gel-free approach allowed the identification of proteins with physicochemical properties (p*I*>10, high molecular weight) that would have hindered their identification through classical gel-based proteomic approaches. K310 identified surface proteins were involved in different cellular processes, such as glycolysis, stress response and protein biosynthesis; for many of them we provided the first evidence of a localisation within the cell wall. The temporal analysis highlighted the dynamic nature of K310 surfome and indicated that such a methodological approach can be applied to study of stress responses in yeast.

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5.7

PROTEOMIC ANALYSIS OF TEMPERATURE-INDUCED CHANGES IN PROTEIN EXPRESSION OF *STENOTROPHOMONAS MALTOPHILIA* K279A REVEALS A ROLE FOR GroEL IN THE HOST ADAPTATION

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Stenotrophomonas maltophilia is emerged as an opportunistic bacterial pathogen that can cause hospital-acquired pneumonia in immunocompromised patients, as well as a frequent colonizer of individuals with cystic fibrosis (CF). Two-dimensional gel electrophoresis, followed by MALDI-TOF mass-spectrometry, was used to profile protein expression in *S. maltophilia* K279a grown at 37 °C with respect to the strain grown at 26 °C in order to identify temperature-regulated proteins that may be important for pathogen-host interaction and virulence. The analysis revealed that host temperature promotes induction of proteins involved in energy generation, heme biosynthesis, translation, transport, and general stress response, besides the chaperone GroEL. In order to characterize the *S. maltophilia groE* genes, we showed that *groES* and *groEL* are arranged in a bicistronic operon whose expression complemented *E. coli* defective in *groEL* or *groES*, and that two promoters σ^{32} and σ^{70} were functional under the heat-shock and normal condition, respectively. Quantitative real-time RT-PCR analysis revealed that *groEL* acts as a heat-shock protein at 37 °C, 42 °C, and, to a lesser extent, at 50 °C. Finally, immunoblot analysis showed that *GroEL* strongly reacted with sera from CF patients infected by *S. maltophilia*, but did not with sera from CF patients without S. maltophilia infection, suggesting important additional functions for the *S. maltophilia* protein.

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5.8

METHICILLIN-SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* (PVL)$^+$ IN HUMAN INFECTIONS

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Long established as a hospital pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA) is now present in the community as a major cause of skin and soft tissue infections. Characteristically, most MRSA strains contain Panton-Valentine leukocidin (PVL) genes, a bicomponent pore-forming toxin with the ability to lyse leukocytes, primarily associated with skin infections such as pustulosis and skin abscesses. Although clinicians are currently concerned primarily with MRSA infections, methicillin-susceptible *S. aureus* (MSSA) infections can present with similar epidemiologic and clinical characteristics. Moreover, according to a large multinational clinical trial, PVL-positive *S. aureus* isolates in Europe are more likely to be MSSA than MRSA [1, 2].

In this study, proteomics was used to compare protein profiles of (PVL)$^+$ MSSA and (PVL)$^-$ MSSA in order to detect proteins and modifications potentially related to infections and toxin production.

Two dimensional electrophoresis was applied on MSSA (PVL)$^+$ and MSSA (PVL)$^-$strains isolated from human serious infections collected from different hospitals.

Image and statistical analysis highlighted several spots differentially expressed in (PVL)$^-$ and (PVL)$^+$ isolates; seven of these proteins were identified by MALDI-TOF. In particular, the over expression of *alkyl hydroperoxide reductase* in MSSA (PVL)$^+$ suggests that the response against oxidative stress may be crucial for pathogenesis and resistance during infection. Our results support the evidence that the resistance against oxidative stress of *S. aureus* is an important factor in the ability of pathogen to survive in hospital, community environment and nasal carriage.

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5.9

PROTEOMIC IDENTIFICATION OF SURFACE PROTEINS INVOLVED IN S.AUREUS ADHESION AND INVASION IMPAIRING BY SERRATIOPEPTIDASE

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The majority of biofilm infections are sustained by *Staphylococcus* spp (70-80%) which are resistant to antibiotics due to sessile phenotype. Agr-neg strains are prevalent (70%) in these emerging infections which necessitate new therapeutical approaches. In biofilms formed by *S. epidermidis* treatment with serratiopeptidase (SPEP), a 50-kDa metallo-protease produced by *Serratia marcescens*, abolishes phenotypic resistance to antibiotics. A systematic proteomic study was then designed in order to identify other surface proteins affected by SPEP treatment.
Four *S. aureus* strains were tested in adhesion and invasion assays after treatment with non-bacteriostatic and non-bactericidal concentrations of SPEP. A significant reduction of *S. aureus* adhesion and invasion in both cell lines was observed. Surface proteins and control cells extracts were fractionated by 1D and 2D SDS-PAGE analysis. Protein bands occurring in the control and disappeared following SPEP treatment were in situ digested and the resulting peptide mixtures were directly analysed by LCMSMS. Mass spectral data were used for protein identification using the Mascot software.
SPEP treatment affects the expression of many different surface proteins. Proteomic investigation of *S. aureus* surface proteins might lead to the definition of the molecular mechanisms of SPEP biologic activity in the restoration of sensitivity to antibiotics of bacterial biofilms. These results strongly support the suggestion to use SPEP or similar compounds as anti-biofilm therapeutics.

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5.10

THE MALDI-TOF TECHNOLOGY AS A TOOL FOR IDENTIFICATION AND TYPING OF YEAST, YEAST-LIKE AND MOULD FUNGI IN CLINICAL MICROBIOLOGY: STRENGTH AND PITFALLS

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Fungal infections are becoming worldwide increasing, resulting in significant morbidity and mortality especially in paediatric populations, immunocompromised and transplanted patients. Particularly the Phyla Ascomycota and Basidiomycota are involved in severe infections in children. The Ascomycota include yeasts of the genera *Candida*, *Saccharomyces*, *Pichia*, and many filamentous fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium*. The Basidiomycota *Cryptococcus neoformans* is an important emerging pathogen. Identification of the increasing diversity of fungal pathogens by conventional methods is often difficult, time-consuming and frequently, for unusual fungi, inconclusive. These constraints have led to increasing interest in MALDI-ToF ICMS (Matrix-assisted laser desorption ionization time-of-flight intact cell mass spectrometry) for rapid and reliable fungal identifications.
A MALDI-ToF MS Biotyper was employed for identification of fungal species from clinical specimens and reference strains. More than four hundred clinical isolates of yeast, yeast-like, and mould fungi, from 300 and 100 diversified specimens, respectively, were grown on solid medium for routinary diagnostic procedures and spectral fingerprinting profile generation. Spectra were acquired and processed to produce: *i)* a customized reference database; *ii)* species identification and; *iii)* typing profiling. Spectral reference profiles were processed by using a MALDI-ToF MS/MS analyzer to perform profile characterization. Identifications were compared to conventional (Vitek-2 for *Candida* spp. and morphology classification for mould) and sequencing-based results to produce concordance data. Typing profiles were processed by computational analysis for clinical or diagnostic correlation index searching.
The MALDI-ToF ICMS seems a reliable tool for fungal diagnosis. However, we need to critically develop appropriate databases and standardized working procedures.

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5.11

ADI PATHWAY AND HISTIDINE DECARBOXYLATION ARE COMPETING ROUTES: A PROTEOMIC EVIDENCE IN L. HILGARDII ISE 5211

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Amine production by amino acid decarboxylation is a common feature used by lactic acid bacteria (LAB) to complement lactic fermentation, since it is coupled with a proton-extruding antiport system intended both to get metabolic energy and to counteract intracellular acidity. Since both malolactic fermentation (MLF) and arginine deiminase (ADI) pathway play analogous roles in LAB, the present investigation was aimed to establish reciprocal interactions between amino acid decarboxylation and each of the two routes referred, in a wine-isolated *L.hilgardii* ISE 5211, whose complete genome is still unknown, able to decarboxylate histidine to histamine.
There is evidence that either malate has no influence on histidine decarboxylase (HDC) biosynthesis nor histidine can affect malolactic enzyme level. Conversely, the expression of ADI route enzymes is negatively modulated by histidine both at their constitutive level and at their arginine-induced level. Similarly arginine causes a reduced expression of HDC at both its basal and its histidine-induced level. The proteomic study also allow us to detect a down-regulation exerted by histidine over sugar metabolism enzymes and over a GroEL stress protein. This, together with the observed reciprocal antagonism between arginine deimination and histidine decarboxylation, can have interesting implications for what concerns lactate, amines, ammonia and ethylcarbamate content of a wine resulting in different health risks.

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5.12

PROTEOMIC ANALYSIS OF *CUPRIAVIDUS METALLIDURANS* CH34: A MODEL FOR SOIL PROTEOMIC STUDIES

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The soil proteome is receiving increasing attention as it can provide information on soil functionality, and because soil is a potential reservoir of proteins of biotechnological interest. The fate of the proteins in soil is complex, as proteins can be hydrolysed, denaturated or adsorbed onto clay minerals or entrapped into organo-mineral complexes. While information is available on the extraction of specific proteins from soil such as glomalin or BT toxin, soil proteomic studies are problematic, mainly due to the lack of protocols to extract and purify protein from soil.
To study the influence of main soil properties such as organic matter content, cation-exchange capacity, content and type clay on soil proteome, we have inoculated the fully sequenced *Cupriavidus metallidurans* CH34 strain into mineral and mineral-organic microcosms simulating a model soil.
Microbial proteins were extracted, quantified, analysed by SDS-PAGE and 2-DE and identified by mass spectrometry. The results indicate that extraction of proteins from soils is strongly influenced by the clay type and organic matter content, possibly due to the different protein domains involved in the adsorption process. We conclude that soil properties can influence the protein extraction efficiency and thus soil proteomics.

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5.13

COMPARATIVE SECRETOME ANALYSIS OF FOUR *BACILLUS CLAUSII* STRAINS

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Bacillus species are frequently used as bacterial workhorses in industrial microbial cultivation for production of probiotic compounds and antibiotics. (Schallmey M, et al., 2004. *Can J Microbiol*, **50:**1-17). Bacterial secretory proteins are known to perform "remote-control" functions, such as the provision of nutrients, detoxification of the environment, and killing of potential competitors. A previous proteomic study carried out in our laboratory examined cell proteins extracted from four closely related isogenic *B. Clausii* strain, used to produce a commercial probiotic preparation and led to the recognition of a number of proteins differentially expressed in the four strains. Here, we report a proteomic study of the secretome, i.e. the complement of extracellular secreted proteins, of the four *B. clausii* strains. The main objective of this study was to characterize the proteins actively secreted including secreted enzyme that can be useful in industry and domestic life and metabolic bottlenecks that can contribute to improve process optimization of their production. The electropherogram of the four *B. clausii* secretomes show more than 400 protein spots separated by two-dimensional gel electrophoresis (2-DE). More pronounced differences were revealed at the expression level of specific proteins, some of which represent the most abundant secreted proteins. The different expression pattern of proteins in the four secretomes demonstrates that the *B. clausii* strains, which are characterized by a notable low level of intraspecific genome diversity, present distinct pattern of protein secretion.

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5.14

THE PROBIOTIC *L. REUTERI* DISPLAYS A PECULIAR PROTEIN PATTERN WHEN GROWN IN A SELENIUM-CONTAINING MEDIUM

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Selenium (Se) is an essential dietary trace element for humans and microorganisms. It generally may be considered an anticarcinogenic and antioxidant agent. Se deficiency is associated with various chronic diseases such as oxidative stress, cardiovascular diseases and some kinds of cancer [1]. The use of Se-enriched yeasts and lactobacilli is an interesting approach to solve selenium-deficiency: these microorganisms, grown in presence of inorganic forms of selenium, are able to convert it into more bio-available organic forms; and so, if added to ice-creams or yogurt, Se is released at gut level.
In this study we focused our attention on *Lactobacillus reuteri* LB2 BM, isolated from human faecal samples, able to accumulate 10 fold more selenium than other strains reported in the literature [2]. Our purpose was to understand, by means of a comparative proteomic approach, if selenium addition could modify protein expression causing variations in the metabolism of the strain. Two-dimensional electrophoresis followed by MALDI-TOF/TOF mass spectrometry was performed on *L. reuteri* grown in a MRS medium fortified with selenium (2mg/l) and in a control condition (same medium without selenium). The analysis was developed both on the cytosolic and the membrane-enriched fraction in the acidic pI range (4-7). From the cytosolic fraction 20 differentially expressed proteins were detected: 15 up-regulated and 5 down-regulated, while the membrane fraction analysis revealed 14 up-regulated proteins and 5 down-regulated.

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5.15

A PROTEOMIC APPROACH TO IDENTIFY DIFFERENTIALLY EXPRESSED PROTEINS INVOLVED IN THE DIMORPHIC TRANSITION OF THE YEAST *P. FERMENTANS*

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The yeast *Pichia fermentans* has an interesting potential as bio-control tool due to its ability to counteract brown rot on apple but becomes a destructive pathogen on peach fruits. *P. fermentans* antagonistic behaviour is associated to yeast-like morphology, conversely its pathogenic behaviour is accompanied by the production of elongated cells, hyphae and pseudohyphae. In order to elucidate the mechanisms involved in the induction of the pseudohyphal pathogenic behaviour a proteomic approach allowing the identification of proteins and post translational modifications putatively involved in the dimorphic transition could be useful. In this context, the aim of the present work is the identification of proteins differentially expressed in yeast-like and pseudohyphal cells using a 2D difference gel electrophoresis and tandem MS.
Crude protein extracts from yeast-like and pseudohyphal DiSAABA 726 *P. fermentans* were separated in the first dimension on 18 cm IPG 4-7 strip and in the second dimension on 12% T polyacrilamide gels. After colloidal Coomassie staining, approximately 500 spots were reproducibly detected on each gel, wherein 15 were differentially expressed. Spots of interest were manually excised from gel and subjected to MS analysis and data base search for protein identification. MS data analysis are still under discussion.

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5.16

PROTEOMIC ANALYSIS IDENTIFIES QUALITY BIOMARKERS FOR COOKED HAM PRODUCTION

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We have characterized the effect of three different brine concentrations (15%, 30%, 45%) on protein exudates released during pig meat processing in the production of cooked ham by a proteomic approach based on a combination of two-dimensional electrophoresis and MALDI TOF analysis. The incorporation of brines, composed of water, salts and flavours, is frequently used to improve meat quality [1]. Brine is mechanically distributed inside the leg via the tumbling process causing extraction of protein. Two dimensional electrophoresis was performed using IPG strips pH 4-7. The peptide mass fingerprinting analysis was primarily focused on the nine most abundant spots common to all exudates and present in quantities allowing confident assignment of protein identities. These common spots account for 90, 88 and 66% of the total averaged spot number for samples at 15%, 30% and 45% brine levels, respectively. The lower intensity spots, whose quantity was insufficient to produce reliable identification, were also closely evaluated. Data demonstrate that brine levels and, hence, composition, dictate the outcome of proteins extraction from the muscles. Distinct proteins and, especially, different quantities of proteins are extracted at defined brine levels. It is known that the composition of protein pattern has different impact on the final texture of at the final product and, hence, on its quality. The detailed molecular analysis of the protein profile, obtained at different brine level, makes possible to identify quality biomarkers to be quickly checked in order to certified quality of final product.

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5.17

DETECTION OF PATHOGENICITY FACTORS IN THE EXTRACELLULAR PROTEOME OF AN HOSPITAL-ISOLATED ENTEROCOCCUS FAECALIS STRAIN

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The interest towards *Enterococcus faecalis* species is increasing because of both its employment in food industries and its impact on human health. The importance of *E. faecalis* in fermented food industry is mainly due to its lypolitic and proteolytic activities useful in cheese industry. On the contrary other strains of this same species are potentially pathogens being often involved in nosocomial infections. Bacterial pathogenicity mainly depends by the secreted molecules that directly interact with the host. For this reason we compare the extracellular proteomes of *E. faecalis* DISAV 1022 and *E. faecalis* H1 isolated from a Piedmont cheese and from an open surgical wound exudate, respectively. Both the strains were grown in M17 medium at 37°C without shaking. Supernatants were recovered in the exponential phase and proteins were precipitated with TCA. Proteomic analyses were performed by two-dimensional electrophoresis coupled with MALDI TOF/TOF mass spectrometry. The results highlighted the presence of proteins linked to pathogenicity only in *E. faecalis* H1: in particular an extracellular serine protease and the coccolysin which are among the main virulence factors of this species, were produced. Furthermore it expresses also a superoxide dismutase and some glycolytic enzymes probably present in extracellular broth as moonlighting proteins which are known to be virulence factors. On the contrary the food strain didn't produce potentially dangerous extracellular proteins. The expression of serine protease and coccolysin in *E. faecalis* H1 were also confirmed by phenotypical assays. Genetic analyses revealed that both the strains possess the genetical determinats for extracellular serine protease and superoxide dismutase, while only *E. faecalis* H1 has the genetical determinant for coccolysin.

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5.18

SUBPROTEOME ANALYSES TO DETECT THE PROTEIN NETWORK INDUCED BY HYDROPHOBIC CARBON SOURCES IN *A. RADIORESISTENS* S13

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Acinetobacter radioresistens S13 is a strain of environmental origin (activated sludge pilot plant) useful from an industrial viewpoint both for bioremediation (hydrocarbon contaminated soils, oil spills, aromatic polluted waters) and for enzymes and surfactant production.
The present investigation is intended to evaluate, by means of 2DE plus mass spectrometry, the global response of the strain to an hydrophobic environment (aromatic compounds as carbon sources in a minimal chemically defined medium, to optimize growth and substrate degradation and control stress). Both soluble (citosolic and periplasmic) and membrane (inner and outer) proteomes have been analyzed in the alkaline pH range and several proteins have been detected by differential expression versus a control condition (acetate medium). The identified proteins can be ascribed to 4 main functional groups: 1) Surface properties modifications 2) Stress responses 3) Energy metabolism 4) pH control.
The results obtained proved to be a good complement to previous investigations on the pI 4 - 7 proteome, allowing the reconstruction of networks of proteins biosynthesized in response to a hydrophobic environment.

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5.19

SELECTIVE IDENTIFICATION OF TRP-CONTAINING PEPTIDES FOR THE ANALYSIS OF ALLERGENIC PROTEINS IN FOODS

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Nowadays the identification of protein allergens in foods is conducted by using immunochemical methods, such as ELISA tests. However, these techniques suffer from several limitations due to cross-reactivity and false negative results [1]. Since allergens are toxic even in trace amounts, there is a need for reliable and sensitive methods for the analysis of allergenic proteins. The purpose of this work was to develop a procedure for identifying these proteins in food samples by using mass spectrometry (MS). We have attempted to detect a target protein contained in a protein extract by the fingerprinting/MS approach upon isolation and MS analysis only of tryptophan (Trp) containing peptides. To this aim, we made use of the selective and quantitative derivatization of Trp residues using the 2,4-dinitrophenyl-sulfenyl chloride (DNPS-Cl) reagent, that leads to a Trp derivative with the DNPS label attached at the 2-position of the indole nucleus [2]. The selection of Trp(DNPS)-peptides from a tryptic digest of a protein sample was achieved by a "diagonal" chromatography approach [3] exploiting the significant change in hydrophobicity and retention time of DNPS-modified peptides in a reverse-phase HPLC column. A protein extract from bovine milk was used to validate the DNPS-derivatization technique and the main protein allergens were thus identified. Alternatively, selective enrichment of DNPS-labeled Trp-peptides was achieved by immunoaffinity chromatography using a column prepared with anti-DNP antibodies, followed by HPLC-MS/MS analysis. Overall, our results and others [4], show that the selective labeling and isolation of Trp-peptides allows a considerable simplification of the fingerprinting/MS approaches nowadays used for the identification of low abundant proteins in proteomics research.

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5.20

NITROGEN DEPRIVATION IN *CHLAMYDOMONAS REINHARDTII*.

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Chlamydomonas reinhardtii is a single celled chlorophyte. Highly adaptable, these green algae live in many different environments throughout the world. Normally, this organism derives energy from photosynthesis; however, *C. reinhardtii* can also thrive in total darkness by using citrate as carbon source for catabolism. Moreover *Chlamydomonas reinhardtii* is being explored as a means to produce biomass and biofuel from wastewater [1]. One of those biofuels involves extracting the cytoplasmic lipid bodies that are produced when *C. reinhardtii* enters stationary phase in liquid culture under nitrogen limiting conditions [2].
In this study, the *C. reinhardtii* proteome was analyzed in normal growth conditions and after 48 hours of nitrogen deprivation. Five biological replicates were analyzed from each experimental group and about 700 proteins were identified from each 2D gel map. Preliminary results showed that in nitrogen deprived samples, the level of glyceradehyde-3-phosphate dehydrogenase, a component of the Photosystem II oxygen-evolving complex protein 2, was increased while the levels for two different isoforms of the photosynthetic enzyme RubisCO and isocitrate lyase were reduced. RubisCO catalyzes the first major step of carbon fixation and its decrease level is the result of the stress condition induced by nitrogen deprivation. Isocitrate lyase is the first enzyme of the glyoxylate cycle allowing these organisms to use fats for the synthesis of carbohydrates and its decrease level is consistent with the high amount of lipids produced during nitrogen deprivation [2].

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5.21

PROTEOMIC ANALYSIS OF THE ACID RESISTANCE IN *GLUCONACETOBACTER HANSENII*

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Acetic acid bacteria (AAB) metabolism is characterised by the distinctive ability to oxydize ethanol to acetic acid. Still the mechanisms of the unique metabolism of AAB, which makes them resistant to low pH (as low as 2.0 to 2.2) and high acetic acid concentrations are not fully known. Here we investigate the changes in global protein expression levels during long-term adaptation of *Gluconacetobacter hansenii* to acetate, as a first step to the characterization of the molecular mechanisms underlying acid resistance. Bacteria were grown both at pH 5.8 and at pH 4.0, proteins extracted from both cultures and run onto 2-DE electrophoresis, followed by MS-MS analysis and database search. Through a differential proteomic approach we identified 86 polypeptidic chains that belong to proteins involved in whole cellular processes. Our data suggest that acid growth condition has a global effect on protein expression and alter the expression of a relatively high number of proteins belonging to important cellular and metabolic pathways. In fact Krebs cycle, lipid metabolism and the process of translation seems to be slow down. Besides, cells grown in presence of acetic acid demonstrate a good adaptation to the acidic environment through the new induction of MinE.

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5.22

A NEW SURFACE PROTEIN OF *STREPTOCOCCUS SUIS* IDENTIFIED BY PROTEOMICS APPROACHES

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Streptococcus suis is a major bacterial swine pathogen worldwide. Among the 35 serotypes described, *S. suis* serotype 2 (SS2) is frequently isolated from a range of serious infections comprising meningitis, pneumonia, septicaemia or arthritis. In addition, SS2 is an important zoonotic agent, affecting people in contact with infected pigs and raising public health concerns on possible outbreaks [1]. Use of the capsular polysaccharide, a major virulence factor, as a vaccine has been hampered by its poor immunogenicity. More recently, surface proteins have received increasing attention since these antigens should have, in principle, a high chance of raising effective immune responses. Several surface□exposed proteins, such as muramidase□released protein, extracellular factor and suilysin, have been tested as vaccines. However many strains lack these antigens, which may result in incomplete coverage when used as a single immunizing agent. Therefore, new potential vaccine candidates must be identified to elicit broad protection against SS2 infection. To this end we identified by a proteomics approach and characterized a new protein, designated Sat, which was expressed on the surface of all examined strains of the swine pathogen *Streptococcus suis*. After immunization, a recombinant Sat fragment significantly protected mice against systemic challenge with *S. suis*, thus demonstrating the potential of this protein as a vaccine candidate.

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5.23

NEW TOOLS FOR QUALITY TRAITS IN MARCHIGIANA MEAT

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Variation in quality of meat is due both to different biological factor (genotype, productivity, sex and age) and environmental and technological factors (composition and nutritional level of diet, rearing, transport and slaughter) acting on animals reared. All these factors interact in a complex way and lead to a high variability of muscular structure that characterizes the qualitative characteristic of meat [1]. Marchigiana is a cattle type that has significant somatic development and it is characterized by a high growth capacity and outstanding precocity. The evolution of the Marchigiana breed into a beef-cattle type has been progressive and significant. The evaluation of quality parameter at a molecular level is fundamental to improve breeding and define quality index in Marchigiana. In this study mono and two dimensional electrophoresis and statistical analysis were applied on 40 samples of *Sternocleidomastoid* muscle from Marchigiana breeds in order to evaluate protein biomarker to select novel parameter of quality and association with type of breeding.

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5.24

BIO-MOLECULAR MARKERS FOR THE TRACEABILITY OF POTATO TUBERS

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Food systems are worldwide changing, resulting in a potential for greater availability of food. Globalization requires intensive agriculture and at the same time creates a growing demand for quality, safety, functionality and sustainability systems. The development and adoption of traceable supply chains could help to reach a unambiguous identification of agro-foods backward to the original supplier and forward to the next step in the full-chain traceability system.

The traceability of product flows has become a basic requirement in quality assurance and provides guarantee to consumers on the origin, location and life history of the product as well as from rising food frauds. An important tool in the characterization of agro-food could be the identification of molecular biomarkers for their reliability.

Potato is one of the most widely cultivated crops in Italy, especially the extra-seasonal varieties. Unfortunately in the last years foreign crops from African countries are coming into European markets without identification and appropriate quality and safety controls.

The aim of the present work is the proteomic characterization of off-season potato (*Solanum tuberosum* L.) varieties grown in Southern Italian regions (Campania, Apulia and Sicily).

A preliminary study was carried out to find a suitable storage method to preserve tuber characteristics after harvesting over time as well as to set a technique of protein extraction to improve the efficiency in terms of quality and quantity. After that two-dimensional gel electrophoresis was used to compare the proteomes of different cultivar tubers to identify factors able to discriminate different cultivars.

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5.25

DEVELOPMENT OF A MASS SPECTROMETRY METHOD TO DETECT RESIDUAL ALLERGENIC FINING PROTEINS IN WINE

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Hidden allergens are a frequent problem for food safety, caused by carry over from previously produced products**,** contaminations from materials used as processing aids, or lack of labelling declaration. The problem of hidden allergens affects winemakers because potential allergenic proteins such as egg albumin and casein are used as fining agents during vinification [1]. Recently also wheat gluten has been authorized as wine clarifying agent [2,3]. These proteins are important allergens and the presence of their residues in the fined wine can not be excluded at levels that may be sufficient to cause clinical symptoms in allergic people.

Nowadays the detection of residual fining agents is based on immunological methods but results of these tests seem to be conflicting [4,5]. Therefore the European Food Safety Authority encourages the development of new sensitive and reliable methods of analysis.

The aim of our study is to develop a new mass spectrometry based method to detect the presence of residual allergenic proteins in wines, including ovalbumin, casein and wheat gluten proteins. We set up the methodology utilizing a model wine matrix and then we applied the optimized method to the analysis of commercial red and white wines. Our results show that it is possible to detect the presence in wine of residues of the fining proteins tested with a sensitivity about ten times higher than that of the conventional immunological approaches.

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APPLICATION OF AN INTEGRATED METABOLOMIC-PROTEOMIC APPROACH TO THE STUDY OF QUORUM-SENSING IN SOURDOUGH LACTOBACILLI

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A new integrated metabolomic-proteomic approach based on ^{1}H-NMR/GC-MS-SPME platform and 2-DE coupled with nano-ESI-MS/MS, respectively, has been developed and applied to investigate the quorum sensing (QS) in sourdough lactobacilli. According to the cell viability decline of *L. sanfranciscensis* DPPMA174 in co-culture with *L. plantarum* DC400, GC-MS-SPME and ^{1}H-NMR profiles obtained in this condition showed dramatic changes in the concentration of several molecules. In particular, when *L. sanfranciscensis* DPPMA174 was co-cultivated with *L. plantarum* DC400, a significant production, of citrate, malate, malonate, decanoic acid and the furanone B has been evidenced. Furanones have been deeply characterized as QS molecules in LAB. Also amino acids such as Asp, Val, Leu, Ileu and Tyr increased in co-culture while molecules associated to bacterial metabolism such as diacetyl and acetoin decreased. In co-culture *Pediococcus pentosaceus* 2XA3 overproduced an interesting metabolite (at 2.52 ppm) whose identification is under investigation by means of ^{1}H-NMR. Compared to mono-culture, the proteome of *L. sanfranciscensis* DPPMA174 grown in co-culture with *L. plantarum* DC400 showed the variation of expression of 58 proteins (47 over expressed and 11 repressed). Fifty-one of the above 58 proteins were identified. They have a central role in stress response, amino acid, energy and nucleotide metabolisms, membrane transport, regulation of transcription, and cell redox homeostasis.

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APPLICATION OF PROTEOMIC TECHNIQUES TO PROTEIN AND PEPTIDE PROFILING OF BOVINE WHEY

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Within the dairy industry it is desirable to control the technological properties of milk to achieve optimal cheese yield and quality. For this reason it's important to know the protein composition of bovine whey before using it in cheese production, and to understand quality, safety and nutritional value of the raw material utilized. In this study a 1-DE, 2-DE LC/MS approach were applied to analyze whey proteins used for ricotta production. Among the major components of whey we have identified HAS, alpha-lactalbumin, beta-lactoglobulin, secretory component, and Ig chains. Besides these major whey components, other proteins were identified which were interesting from a nutriceutical point of view (such as lactoferrin, and lactotransferrin). We have also demonstrated that the amount of beta-lactoglobulin had positive effects on the yield of ricotta made from the individual milk samples. In addition we have performed Tris-Tricine-SDS-PAGE analysis combined with MS compatible silver staining, in order to completely characterized the bioactive peptides present in milk whey.

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1.66 CHARACTERIZATION OF TWO ISOFORMS OF HUMAN SPRR3 FROM HUMAN PRETERM NEWBORN SALIVA, AUTOPTIC FETAL ORAL MUCOSA AND PAROTID GLAND SAMPLES
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1.68 CONTRIBUTION OF URINE PROTEOME ANALYSIS FOR NON-INVASIVE DIFFERENTIAL DIAGNOSIS OF DIABETIC NEPHROPATHY
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1.69 IDENTIFICATION OF FERTILITY BIOMARKERS BY 2D-SDS PAGE AND MALDI - TOF / TOF
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1.70 DIFFERENTIAL PROTEOMICS OF RCC URINARY EXOSOME: AN UPDATE
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5.9 PROTEOMIC IDENTIFICATION OF SURFACE PROTEINS INVOLVED IN S.AUREUS ADHESION AND INVASION IMPAIRING BY SERRATIOPEPTIDASE
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